DISTRIBUTION, DIVERSITY AND FATE OF *SALMONELLA* IN NATURAL BIOFILMS

DISSERTATION

Presented to the Graduate Council
of Texas State University-San Marcos
in Partial Fulfillment
of the Requirements

for the Degree

Doctor of PHILOSOPHY

by

Qiong Sha, M.S.

San Marcos, Texas
December 2012

DISTRIBUTION, DIVERSITY AND FATE OF *SALMONELLA* IN NATURAL BIOFILMS

Committee members approved:

_____

Dittmar Hahn, Chair

_____

Robert J. C. Mclean

_____

Floyd Weckerly

_____

Charles P. Gerba

_____

Jorge Rodrigues

Approved:

_____

J. Michael Willoughby
Dean of the Graduate College

FAIR USE AND AUTHOR'S PERMISSION STATEMENT

Fair Use

Duplication Permission

ACKNOWLEDGEMENTS

The author is grateful for the professional advice from her committee members Dr. Hahn, Dr. McLean, Dr. Weckerly, Dr. Gerba and Dr. Rodrigues, as well as for the financial support from Texas State University-San Marcos, the Department of Biology, the Alexander-Stone endowment, and the State of Texas through the American Recovery and Reinvestment Act (ARRA). The author thanks her friends James (Buddy) Gaertner, Joseph Mendoza, Zanxun Dai, Leanna McMillin, Michael Bitzer and Jacqueline Hernandez for their help during the experiments. The author is also indebted to her family for their unconditional love and support. She also thanks her labmates and friends for their general support during her Ph.D. study.

This manuscript was submitted on October 15, 2012.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

DISTRIBUTION, DIVERSITY AND FATE OF *SALMONELLA* IN NATURAL BIOFILMS

by

Qiong Sha, M.S.

Texas State University-San Marcos

December 2012

SUPERVISING PROFESSOR: DITTMAR HAHN

Salmonella enterica strains represent important enteric pathogens that are typically transmitted to humans via food and drinking water contaminated with feces of vertebrate animals. The intestinal tract of vertebrates is typically presumed to be the native habitat of salmonellae, however, recent studies frequently detected *Salmonella* strains in water, sediments, animals (i.e., fish, turtles) and biofilms even in supposedly clean habitats such as Spring Lake, the spring-fed headwaters of the San Marcos River, Texas. We therefore proposed to monitor these potential human pathogens as they persist in or move through such ecosystems using a combination of traditional enrichment culture techniques in combination with molecular detection and identification tools that allow for highly sensitive, high-resolution analyses of salmonellae.

An initial study [Chapter 2, published in Systematic and Applied Microbiology 34, 353-359 (2011)] assessed the diversity and distribution of salmonellae in freshwater biofilms at a fine scale (i.e. in 20 locations from a 324 cm^2 area) for two sites in San Marcos, TX, a concrete storm water overflow channel (City Park) and a concrete surface in the spring-fed headwaters of the San Marcos River (Spring Lake) between April and September 2009. The study demonstrated the presence of salmonellae in natural biofilms and a significant micro-heterogeneity with differences in diversity and persistence of salmonellae during the season. The composition of *Salmonella* strains in the area analyzed changed in time with large differences between early (April, June) and late sampling times (September) within and among sites, except for one strain (S12) that was present at almost all sampling times at both sites, though often at different locations within the area analyzed.

Follow-up studies [Chapter 3, published in Microbial Ecology DOI: 10.1007/s00248-012-0106-y (2013)] identified 4 selected strains as serovars Give, Thompson, Newport and -:z10:z39, and confirmed their pathogenicity in feeding studies with the nematode *Caenorhabditis elegans* demonstrating that pathogenic salmonellae were isolated from heterogeneous aquatic biofilms. Cells of these isolates inoculated into water or biofilms declined numerically within 2 days, reaching the detection limit of our *q*PCR-based quantification technique (i.e. 10^3 cells ml^{-1}); however, cells persisted and stayed viable in biofilms in high numbers for some time.

The fourth chapter [accepted by FEMS Microbiology Ecology] focused on the analyses of the diversity of *Salmonella* in biofilm and water samples from the spring and slough arms of Spring Lake during the drought of 2011, with only one potential run-off event at the beginning of the study. Salmonellae were detected in semi-selective enrichment cultures by end-point PCR during the entire sampling period (11 sampling events during 2 months). From the spring arm site, 73% of the biofilms and 41% of the water samples were positive for salmonellae, while only 9% of the biofilms and 23% of the water samples were positive from the slough arm site. Salmonellae could be isolated from all positive samples, with higher diversity in biofilms compared to water samples, and more strains obtained from the spring arm than from the slough arm. Differences between sites were generally caused by less frequently detected isolates, while the majority of isolates that were present in both biofilms and water from both sites was represented by three strains only. Quantification attempts by *q*PCR directly in samples without prior enrichment did not result in a reliable detection of salmonellae, suggesting that numbers in all samples were below the detection limit.

One of the strains isolated from biofilms was used to assess the potential of fish to transfer salmonellae from heterogeneous aquatic biofilms into feces using controlled aquarium studies with suckermouth catfish (*Hypostomus plecostomus*) and biofilms on tiles inoculated with salmonellae [Chapter 5]. Neither the presence of fish nor inoculation with salmonellae had detectable effects on the abundance of the microbial community, i.e. all DAPI-stained cells. Numbers of salmonellae quantified by *q*PCR and by *in situ*

hybridization in water and biofilms, however, decreased fast from an initial value representing about 20% of the DAPI-stained cells to less than 0.01% within 3 days indicating that salmonellae are not persisting in high numbers in these environments, but probably present in low numbers.

The results presented in this thesis indicate long-term persistence of *Salmonella* at considerable diversity, albeit in low numbers, in both water and heterogeneous aquatic biofilms, even in the absence of concurrent runoff that could be expected to contribute to contamination.

CHAPTER 1

General Introduction

Salmonellae are a group of gram negative bacteria recognized as major zoonotic pathogens worldwide for both humans and animals (Humphrey, 2000). Salmonellae are of great public concern because they can cause intestinal diseases such as gastroenteritis (i.e., salmonellosis), and are responsible for 1.3 billion cases annually worldwide (Pang, *et al.*, 1995). In the United States alone, 1.4 million people are infected by *Salmonella* strains resulting in costs of half a billion dollar annually based on medical care costs and lost productivity (Frenzen, *et al.*, 1999). Most of the infections are caused by the consumption of contaminated, uncooked animal products or raw food (Tauxe, 1997, Mutangadura, 2004), although infections through contaminated water have been reported frequently (Angulo, *et al.*, 1997, Van Houten, *et al.*, 1998, O'Reilly, *et al.*, 2007, Haley, *et al.*, 2009). The intestinal tracts of warm- and many cold-blooded animals are considered to be the natural habitat of salmonellae (Woodward, *et al.*, 1997), which is supported by the detection of this pathogen in a variety of animals, such as birds (Refsum, *et al.*, 2002, Iveson, *et al.*, 2009, Phalen, *et al.*, 2010), reptiles (Woodward, *et al.*, 1997, Briones, *et al.*, 2004, Hahn, *et al.*, 2007, Gaertner, *et al.*, 2008), mammals (Tejedor-Junco, *et al.*, 2009), and fish (Wyatt, *et al.*, 1979, Lawton & Morse, 1980, Gaertner, *et al.*, 2008). However,

salmonellae have also been found in environments like sediments (Moore, *et al.*, 2003, Martinez-Urtaza, *et al.*, 2004), soil (Cote & Quessy, 2005, Danyluk, *et al.*, 2008) and water (Cherry, *et al.*, 1972, Cherry, *et al.*, 1975, Jiménez, *et al.*, 1989, Martinez-Urtaza, *et al.*, 2004). Numerous studies have been conducted on salmonellae and their hosts from the pathogen control point of view, however, little is known about the fate of this pathogen outside their hosts. The fact that salmonellae have been recovered from rivers and streams in remote areas without any influence from humans (Fair & Morrison, 1967, Hendricks & Morrison, 1967, Thomason, *et al.*, 1975) and from non-symptomatic animal carriers (Hendrick.Cw, 1971, Chao, *et al.*, 1987) suggests that the interaction between this organism and the environments might be much more complex than people used to think. The paradigm for salmonellae as a contaminant in the environment therefore needs to be refined, and the potential of this organism as an ecosystem component be investigated.

Objectives

Previously, salmonellae have been detected from natural biofilms (Gaertner, *et al.*, 2009, Gaertner, *et al.*, 2011), algae mats (Ishii, *et al.*, 2006, Englebert, *et al.*, 2008, Byappanahalli, *et al.*, 2009, Gaertner, *et al.*, 2009, Gaertner, *et al.*, 2011) and the biofilms on the carapace of turtles (Gaertner, *et al.*, 2008, Gaertner, *et al.*, 2008). These studies suggested that biofilms and algae might support the persistence of salmonellae in aquatic systems after a non-point contamination incidence. Therefore,

we hypothesized that biofilms could serve as a reservoir for salmonellae survival, long-term persistence and even growth as was suggested for other pathogens (Watnick & Kolter, 1999, Yildiz & Schoolnik, 1999, Topp*, et al.*, 2003).

The objective of this study was therefore to monitor the potential human pathogen *Salmonella* as it moves through non-intestinal ecosystems using a combination of traditional enrichment culture techniques in combination with molecular detection and identification tools that allow for highly sensitive, high-resolution analyses of salmonellae. This study addressed several hypotheses that focused on the analyses of the distribution, the dissemination and the short- and long-term establishment of salmonellae in water and biofilms of a pristine aquatic habitat (i.e., Spring Lake, San Marcos, TX) and adjacent areas:

1. salmonellae are distributed randomly with high diversity in biofilms in natural aquatic systems,
2. biofilms provide habitat suitable for long-term persistence and potential growth of salmonellae in aquatic systems,
3. biofilms represent potential reservoirs for the distribution of salmonellae into the food chain,
4. salmonellae contamination in natural environments could be from animal feces through precipitation runoffs,
5. individual strains persist long-term in aquatic environments in biofilms and/or animal reservoirs.

In order to test these hypotheses, 3 sites were chosen around Spring Lake, the head waters of the San Marcos River in San Marcos (TX, USA) including: the spring arm of Spring Lake which is fed by spring waters and was considered to be the most pristine aquatic ecosystem in Texas (Slattery & Fahlquist, 1997); the slough arm of Spring Lake which is connected to the Sink Creek discharge area in the middle of a golf course; and the City Park site which resembled a storm water overflow channel, connecting several ponds on the campus of Texas State University-San Marcos with the San Marcos River (Fig. 1.1). The ecology of salmonellae was investigated at these sites through a series of experiments that included both field sampling, and mesocosm studies performed to eliminate complex environmental effects and to focus on one particular variable, such as the persistence ability of salmonellae in natural biofilms. In addition, pathogenicity tests were included to demonstrate that *Salmonella* strains retrieved from natural biofilms were virulent. Based on the preliminary results on the distribution and diversity of *Salmonella* in natural aquatic environments, quantitative studies were conducted in follow-up experiments. In order to quantify *Salmonella* in natural biofilms, biofilms were allowed to develop on ceramic tiles with defined surface area initially and further used for salmonellae quantitative studies. Animal feces were suggested to provide contamination sources for *Salmonella* in natural environments in numerous studies (Thomason, *et al.*, 1975, Polo, *et al.*, 1998, Refsum, *et al.*, 2002, Tavechio, *et al.*, 2002, Martinez-Urtaza, *et al.*, 2004). Thus, we were interested to see whether fish [i.e., suckermouth catfish (*Hypostomus plecostomus*)]

were able to transfer salmonellae from biofilms into feces, and whether these feces could be a potent source of release and contamination of salmonellae.

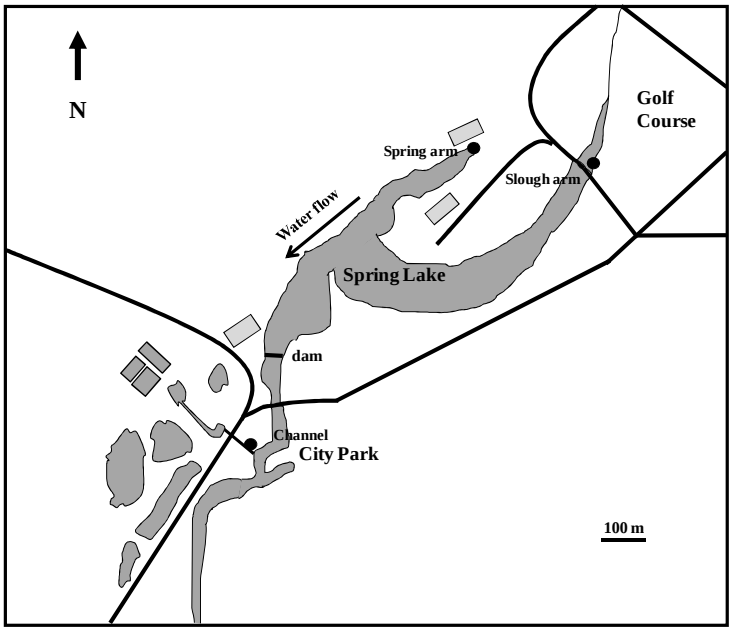


Fig. 1.1 Schematic presentation of sampling sites

The final goal of this study was to establish quantitative information on the distribution, dissemination and fate of salmonellae in water and biofilms of Spring Lake as a proxy for aquatic systems generally, and then evaluate the potential consequences for spread and establishment of these pathogenic bacteria. Significant baseline data on the fate of an obviously widespread, but little-studied organism outside potential hosts and clinical environments will be established and used to discuss routes of contamination and population establishment using sensitive, high resolution methods that are basis for reliable studies on the epidemiology of pathogens like salmonellae in the environment.

References

[1] Angulo FJ, Tippen S, Sharp DJ, *et al.* (1997) A community waterborne outbreak of salmonellosis and the effectiveness of a boil water order. *American Journal of Public Health* **87**: 580-584.

[2] Briones V, Sonia T, Joaquín G, Cristina B, María del Pilar L, Lucas D & José FF-G (2004) *Salmonella* diversity associated with wild reptiles and amphibians in Spain. *Environmental Microbiology* **6**: 868-871.

[3] Byappanahalli MN, Sawdey R, Ishii S, Shively DA, Ferguson JA, Whitman RL & Sadowsky MJ (2009) Seasonal stability of *Cladophora*-associated *Salmonella* in Lake Michigan watersheds. *Water Research* **43**: 806-814.

[4] Chao WL, Ding RJ & Chen RS (1987) Survival of pathogenic bacteria in environmental microcosms. *Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi* **20**: 339-348.

[5] Cherry WB, Thomason BM, Gladden JB, Holsing N & Murlin AM (1975) Detection of *Salmonella* in foodstuffs, feces, and water by immunofluorescence *Annals of the New York Academy of Sciences* **254**: 350-368.

[6] Cherry WB, J. B. Hanks, B. M. Thomason, A. M. Murlin, J. W. Biddle a & Croom JM (1972) Salmonellae as an index of pollution of surface waters. *Applied Microbiology.* **24**: 334–340.

[7] Cote C & Quessy S (2005) Persistence of *Escherichia coli* and *Salmonella* in surface soil following application of liquid hog manure for production of pickling cucumbers. *Journal of Food Protection* **68**: 900-905.

[8] Danyluk MD, Nozawa-Inoue M, Hristova KR, Scow KM, Lampinen B & Harris LJ (2008) Survival and growth of *Salmonella* Enteritidis PT 30 in almond orchard soils. *Journal of Applied Microbiology* **104**: 1391-1399.

[9] Englebert ET, McDermott C & Kleinheinz GT (2008) Impact of the alga *Cladophora* on the survival of *E-coli*, *Salmonella*, and *Shigella* in laboratory microcosm. *Journal of Great Lakes Research* **34**: 377-382.

[10] Fair JF & Morrison SM (1967) Recovery of bacterial pathogens from high quality surface water. *Water Resources Research* **3**: 799-803.

[11] Frenzen PD, Riggs TL, Buzby JC, Breuer T & Roberts T (1999) *Salmonella* cost estimate updated using foodnet data. *Food Safety* **22**: 10-15.

[12] Gaertner J, Wheeler PE, Obafemi S, Valdez J, Forstner MRJ, Bonner TH & Hahn D (2008) Detection of salmonellae from fish in a natural river system. *Journal of Aquatic Animal Health* **20**: 150-157.

[13] Gaertner JP, Hahn D, Rose FL & Forstner MRJ (2008) Detection of salmonellae in different turtle species within a headwater spring ecosystem. *Journal of Wildlife Diseases* **44**: 519-526.

[14] Gaertner JP, Mendoza JA, Forstner MRJ & Hahn D (2011) Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. *Journal of Water and Health* **9**: 458-466.

[15] Gaertner JP, Hahn D, Jackson J, Forstner MRJ & Rose FL (2008) Detection of salmonellae in captive and free-ranging turtles using enrichment culture and polymerase chain reaction. *Journal of Herpetology* **42**: 223-231.

[16] Gaertner JP, Garres T, Becker JC, Jimenez ML, Forstner MRJ & Hahn D (2009) Temporal analyses of salmonellae in a headwater spring ecosystem reveals the effects of precipitation and runoff events. *Journal of Water and Health* **7**: 115-121.

[17] Hahn D, Gaertner J, Forstner MRJ & Rose FL (2007) High-resolution analysis of salmonellae from turtles within a headwater spring ecosystem. *FEMS Microbiology Ecology* **60**: 148-155.

[18] Haley BJ, Cole DJ & Lipp EK (2009) Distribution, diversity, and seasonality of waterborne salmonellae in a rural watershed. *Applied and Environmental Microbiology* **75**: 1248-1255.

[19] Hendrick.Cw (1971) Increased recovery rate of salmonellae from stream bottom sediments versus surface waters. *Applied Microbiology* **21**: 379-380.

[20] Hendricks CW & Morrison SM (1967) Multiplication and growth of selected enteric bacteria in clear mountain stream water. *Water Research.* **1**: 567-576.

[21] Humphrey T (2000) *Public-health aspects of Salmonella infection*. CABI Publishing, Wallingford, UK.

[22] Ishii S, Yan T, Shively DA, Byappanahalli MN, Whitman RL & Sadowsky MJ (2006) *Cladophora* (Chlorophyta) *spp*. harbor human bacterial pathogens in nearshore water of Lake Michigan. *Applied and Environmental Microbiology* **72**: 4545-4553.

[23] Iveson JB, Shellam GR, Bradshaw SD, Smith DW, Mackenzie JS & Mofflin RG (2009) *Salmonella* infections in Antarctic fauna and island populations of wildlife exposed to human activities in coastal areas of Australia. *Epidemiology and Infection* **137**: 858-870.

[24] Jiménez L, Muñiz I, Toranzos GA & Hazen TC (1989) Survival and activity of *Salmonella typhimurium* and *Escherichia coli* in tropical freshwater. *Journal of Applied Microbiology* **67**: 61-69.

[25] Lawton RL & Morse EV (1980) *Salmonella* survival in freshwater and experimental infections in goldfish (*Crassuis auratus*). *Journal of Environmental Science and Health . Part A: Environmental Science and Engineering* **15**: 339-358.

[26] Martinez-Urtaza J, Saco M, de Novoa J, Perez-Pineiro P, Peiteado J, Lozano-Leon A & Garcia-Martin O (2004) Influence of environmental factors and human activity on the presence of salmonella serovars in a marine environment. *Applied and Environmental Microbiology* **70**: 2089-2097.

[27] Martinez-Urtaza J, Saco M, de Novoa J, Perez-Pineiro P, Peiteado J, Lozano-Leon A & Garcia-Martin O (2004) Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. *Applied and Environmental Microbiology* **70**: 2089-2097.

[28] Moore BC, Martinez E, Gay JM & Rice DH (2003) Survival of *Salmonella enterica* in freshwater and sediments and transmission by the aquatic midge *Chironomus tentans* (Chironomidae: Diptera). *Applied and Environmental Microbiology* **69**: 4556-4560.

[29] Mutangadura GB (2004) World health report 2002: Reducing risks, promoting healthy life. *Agricultural Economics* **30**: 170-172.

[30] O'Reilly CE, Bowen AB, Perez NE, *et al.* (2007) A waterborne outbreak of gastroenteritis with multiple etiologies among resort island visitors and residents: Ohio, 2004. *Clinical Infectious Diseases* **44**: 506-512.

[31] Pang T, Bhutta ZA, Finlay BB & Altwegg M (1995) Typhoid-fever and other salmonellosis - a continuing challenge. *Trends in Microbiology* **3**: 253-255.

[32] Phalen DN, Drew ML, Simpson B, Roset K, Dubose K & Mora M (2010) *Salmonella enterica* subsp. *enterica* in cattle egert (*Bubulcus ibis*) chicks from central Texas: prevalence, serotypes, pathogenicity, and epizootic potential. *Journal of Wildlife Diseases* **46**: 379-389.

[33] Polo F, Figueras MJ, Inza I, Sala J, Fleisher JM & Guarro J (1998) Relationship between presence of *Salmonella* and indicators of faecal pollution in aquatic habitats. *FEMS Microbiology Letters* **160**: 253-256.

[34] Refsum T, Heir E, Kapperud G, Vardund T & Holstad G (2002) Molecular epidemiology of *Salmonella enterica* serovar Typhimurium isolates determined by pulsed-field gel electrophoresis: comparison of isolates from avian wildlife, domestic animals, and the environment in Norway. *Applied and Environmental Microbiology* **68**: 5600-5606.

[35] Renter DG, Gnad DP, Sargeant JM & HygnstroM SE (2006) Prevalence and serovars of *Salmonella* in the feces of free-ranging white-tailed deer (*Odocoileus virginianus*) in Nebraska. *Journal of Wildlife Diseases* **42**: 699-703.

[36] Slattery RN & Fahlquist L (1997) Water quality summary of the San Marcos Springs Riverine System, San Marcos, Texas, July-August 1994. Vol. FS-059-57, San Antonio, TX.

[37] Tauxe RV (1997) Emerging foodborne diseases: An evolving public health challenge. *Emerging Infectious Diseases* **3**: 425-434.

[38] Tavechio AT, Ghilardi ACR, Peresi JTM, Fuzihara TO, Yonamine EK, Jakabi M & Fernandes SA (2002) *Salmonella* serotypes isolated from nonhuman sources in Sao Paulo, Brazil, from 1996 through 2000. *Journal of Food Protection* **65**: 1041-1044.

[39] Tejedor-Junco MT, Lupiola P, Caballero MJ, Corbera JA & Gutierrez C (2009) Multiple abscesses caused by *Salmonella enterica* and *Corynebacterium pseudotuberculosis* in a dromedary camel. *Tropical Animal Health and Production* **41**: 711-714.

[40] Thomason BM, Biddle JW & Cherry WB (1975) Detection of salmonellae in the environment. *Applied and Environmental Microbiology* **30**: 764-767.

[41] Thomason BM, Biddle JW & Cherry WB (1975) Detection of *salmonellae* in environment. *Applied Microbiology* **30**: 764-767.

[42] Topp E, Welsh M, Tien YC, Dang A, Lazarovits G, Conn K & Zhu H (2003) Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. *FEMS Microbiology Ecology* **44**: 303-308.

[43] Van Houten R, Farberman D, Norton J, Ellison J, Kiehlbauch J, Morris T & Smith P (1998) *Plesiomonas shigelloides* and *Salmonella* serotype Hartford infections associated with a contaminated water supply - Livingston County, New York, 1996 (Reprinted from MMWR, vol 47, pg 394-396, 1998). *Infections in Medicine* **15**: 495-497.

[44] Watnick PI & Kolter R (1999) Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Molecular Microbiology* **34**: 586-595.

[45] Woodward DL, Khakhria R & Johnson WM (1997) Human salmonellosis associated with exotic pets. *Journal of Clinical Microbiology* **35**: 2786-2790.

[46] Wyatt LE, Nickelson R & Vanderzant C (1979) Occurence and control of *Salmonella* in freshwater catfish. *Journal of Food Science* **44**: 1067-1073.

[47] Yildiz FH & Schoolnik GK (1999) Vibrio cholerae O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 4028-4033.

CHAPTER 2

TEMPORAL ANALYSES OF THE DISTRIBUTION AND DIVERSITY OF *SALMONELLA* IN NATURAL BIOFILMS

Sha, Q., A. Gunathilake, M.R.J. Forstner, D. Hahn. 2011. Temporal analyses of the distribution and diversity of *Salmonella* in natural biofilms. Systematic and Applied Microbiology **34**(5): 353-359.

Abstract

The diversity and distribution of salmonellae in biofilms were analyzed at a fine scale (i.e. in 20 locations from a 324 cm^2 area) for two sites in San Marcos, TX. A concrete storm water overflow channel (City Park) was sampled 4 times and a concrete surface in the spring-fed headwaters of the San Marcos River (Spring Lake) 5 times between April and September 2009, and each biofilm sample analyzed by a combination of traditional enrichment methods and molecular techniques. PCR detection of *inv*A gene fragments after semi-selective enrichment of salmonellae was achieved in biofilms from all 20 locations at the City Park site, with locations generally being positive 2 to 3 times out of 4 sampling times for a total of 59% positive samples. *Inv*A gene fragment detection in biofilms was less frequent for the 5 sampling times and 20 locations from the Spring Lake site (18% of all samples), with 1 sampling time being entirely negative and 8 locations remaining negative throughout the study. Rep-PCR fingerprinting of 491 *Salmonella* isolates obtained from both sites resulted in 30 distinct profiles, with 26 and 7 profiles retrieved from City Park and Spring Lake samples, respectively, and thus with 3 profiles present at both sites, and multiple strains frequently obtained from single locations at both sites. The composition of *Salmonella* strains in the area analyzed changed in time with large differences between early (April, June) and late sampling times (September) within and among sites, except for one strain (S12) that was abundant at almost all sampling times at both sites, though often at different locations within the area analyzed. These results

demonstrate the presence of salmonellae in natural biofilms and a significant micro-heterogeneity with differences in diversity and persistence of salmonellae.

Introduction

Salmonellae represent a group of gram-negative bacteria that are recognized worldwide as major zoonotic pathogens for both humans and animals (Humphrey, 2000). Salmonellosis affects more people than any other single disease (Turnbull, 1979), with the majority of illnesses resulting from exposure to undercooked animal products or to cross-contaminated foods consumed raw (Tauxe, 1997, Organization, 2002). However, salmonellosis can also result from direct contact with contaminated water (Foltz, 1969, Harvey, *et al.*, 1969) or infected animals (Sanyal, *et al.*, 1997, Wells, *et al.*, 2004, Nakadai, *et al.*, 2005). The native habitat of salmonellae is considered to be the intestinal tract of a taxonomically diverse group of vertebrates (Gray, 1995, Refsum, *et al.*, 2002, Briones, *et al.*, 2004) from which salmonellae can spread to other environments through released feces (Baudart, *et al.*, 2000, Islam, *et al.*, 2004, Chandran & Hatha, 2005, Haley, *et al.*, 2009). However, salmonellae have also been recovered from rivers and streams in remote areas, without detectable impact by humans (Fair & Morrison, 1967, Hendricks & Morrison, 1967, Thomason, *et al.*, 1975) or host animals (Hendrick, 1971, Chao, *et al.*, 1987). This suggests more complex interactions of salmonellae with the environment than indicated by a scenario linking their presence entirely to environmental contamination through, e.g., manure or wastewater discharges (Polo, *et al.*, 1998, Martinez-Urtaza, *et al.*, 2004).

Salmonellae have been shown to survive for extended periods of time in non-enteric habitats (Turpin, *et al.*, 1993, Chandran & Hatha, 2005, Cote & Quessy, 2005, Semenov, *et al.*, 2009), including biofilms and algal mats (Ishii, *et al.*, 2006, Englebert, 2008, Byappanahalli, *et al.*, 2009). Mats of the green algae *Cladophora*, for example, were identified as reservoirs of salmonellae, with isolates exhibiting a high degree of genetic relatedness (Ishii, *et al.*, 2006, Byappanahalli, *et al.*, 2009). These studies suggested a casual relationship between salmonellae and *Cladophora* potentially related to input sources, e.g. runoff, with a predominant genotype surviving on the algae (Byappanahalli, *et al.*, 2009). Runoff was also suggested as the major source of contamination with salmonellae in Spring Lake, San Marcos, Texas (Gaertner, *et al.*, 2009), with a few predominant genotypes of salmonellae establishing in biofilms on the carapace of turtles (Gaertner, *et al.*, 2008) or on concrete surfaces (Gaertner et al., unpublished). From this perspective, biofilms might provide habitats suitable for long-term survival of salmonellae once introduced into aquatic systems, as seen for other pathogens (Watnick & Kolter, 1999, Yildiz & Schoolnik, 1999), or might even support growth as suggested for other environments such as soil (Topp, *et al.*, 2003, You, *et al.*, 2006).

The aim of this study was to assess the presence and establishment of viable salmonellae in biofilms on concrete surfaces at 2 sites, i.e., Spring Lake, the spring-fed headwaters of the San Marcos river, and City Park, a stormwater overflow channel, both in San Marcos, Texas. Biofilms were collected from twenty locations in

an area covering about 324 cm^2 several times during the year 2009, and analyzed for the occurrence and diversity of salmonellae by using a combination of traditional enrichment culture techniques and molecular analysis tools.

Material and Methods

Sampling sites

Biofilm samples were collected at Spring Lake (29.894132, -97.929838), the spring-fed headwaters of the San Marcos River, Texas, USA, and at City Park (29.886579, -97.936171), a stormwater overflow channel connecting several ponds on campus of Texas State University-San Marcos with the San Marcos River about 2 km downstream of Spring Lake (Fig. 2.1). At both sites, biofilms were permanently covered with a thin layer of water, exposed to sunlight at Spring Lake but in the shade at City Park. Samples were meant to be taken about one week after significant rainfall, i.e. usually thunderstorms with heavy precipitation, except for the last sampling that was performed directly after rainfall. From Spring Lake, samples were obtained on April 24, May 5, May 20, September 21 and September 25, and from City Park on June 2, June 22, September 21 and September 25 (Fig. 2.2).

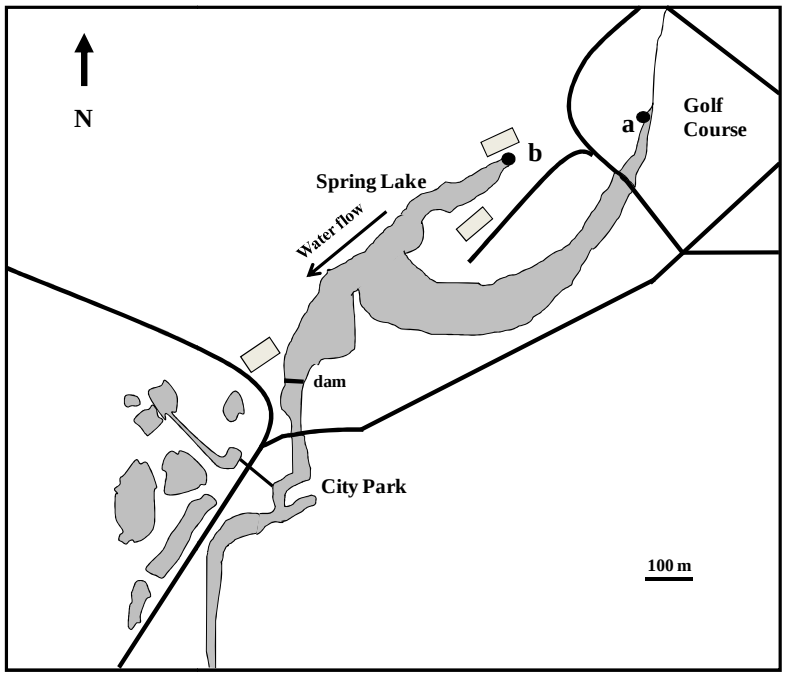


Fig. 2.1 Schematic presentation of sampling sites Spring Lake, the headwaters of the San Marcos River, Texas, USA (29.894132, -97.929838) (a), and City Park, a storm water overflow channel connecting several ponds on campus of Texas State University-San Marcos with the San Marcos River about 2 km downstream of Spring Lake (29.886579, -97.936171). Dark lines represent roads and squares buildings.

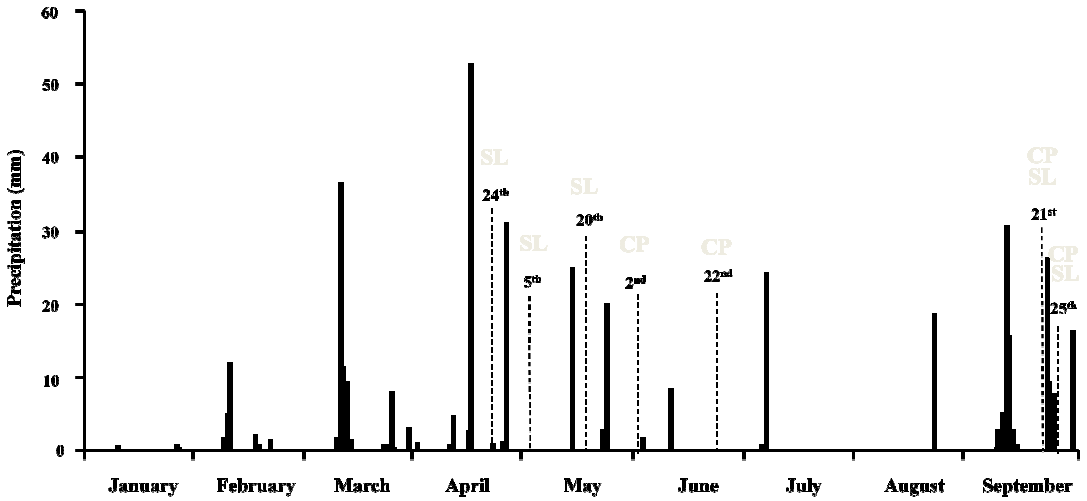


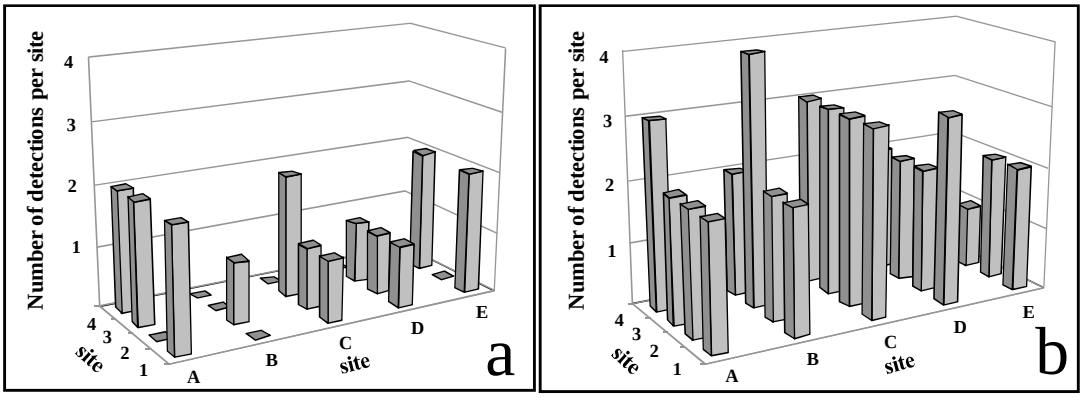
Fig. 2.2 Precipitation data (dark bars, obtained at http://www.ncdc.noaa.gov/oa/ncdc.html for San Marcos, TX, station 417983), sampling dates (dashed lines) and sampling sites (SL, Spring Lake; CP, City Park) for the first nine months of 2009.

At each sampling time, biofilms were retrieved from the concrete surface at the same position with a cork corer (2 cm diameter) and a spatula from a small area (324 cm^2, 12 x 27 cm) in a sampling grid that consisted of 4 rows (1 – 4) and 5 columns (A – E) for a total of 20 locations (A1 – E4) (Fig. 2.3). Samples that were collected in 50 ml Falcon tubes differed from each other with respect to biofilm mass and area as well as to water content due to difficulties in sampling quantitatively from the rough concrete surface under water. Additional water samples, i.e. 40 ml for Spring Lake and 20 ml for City Park (n=3 each) were collected directly into 50 ml Falcon tubes. All samples were processed within an hour after sampling.

Enrichment

Biofilm and water samples were centrifuged (2,000 x g, 15 minutes), and the pellets dispersed into 7 ml sterile distilled water. Six 1-ml sub-samples were centrifuged (14,000 x g, 5 minutes), and the supernatants removed. Three pellets were frozen and stored at -80ºC for potential use in nucleic acid based detection procedures, while the remaining three cell pellets were dispersed in 1 ml of Buffered Peptone Water (BPW; L^{-1}: 10 g peptone, 5 g NaCl, 9 g Na_2HPO_4, 1.5 g KH_2PO_4, pH 7.2) (International Standard Organization, 1993) and incubated at 37ºC for 24 hours. After incubation, 100 µl of these cultures were transferred to 2-ml cryo-tubes containing 1 ml of Rappaport–Vassiliadis (RVS) broth (L^{-1}: 4.5 g soybean peptone, 29 g $MgCl_2 \cdot 7\ H_2O$, 8 g NaCl, 0.4 g K_2HPO_4, 0.6 g KH_2PO_4, 0.036 g malachite-green, pH 5.2) semi-selective for salmonellae (Vassiliadis, *et al.*, 1981) and incubated at 37ºC for 48

hours. Sub-samples (100 µl) were then transferred to cryo-tubes with fresh RVS medium for a second enrichment at 37ºC for 48 hours.



Spring Lake **City Park**

Fig. 2.3 PCR detection of *Salmonella* in enrichments from 20 biofilm samples from Spring Lake and City Park. Each square represents a sample area of about 3 cm^2 (distance from the next site was 1.5 cm (sites 1-4), or 4 cm (sites A-E)).

PCR-based detection

For PCR-based detection of salmonellae, cells in sub-samples (100 µl) of the second enrichment in RVS were pelleted by centrifugation (14,000 x g, 5 minutes), dispersed in 100 µl of 50 mM NaOH and lysed by incubation at 65ºC for 30 minutes. Detection of salmonellae by PCR was based on an established protocol using primers 139 ($^{5'}$GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 ($^{5'}$TCA TCG CAC CGT CAA AGG AAC C) (Rahn, *et al.*, 1992) to amplify a 284-bp-fragment of the *inv*A gene that encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by salmonellae (Suárez & Rüssmann, 1998, Khan, *et al.*, 2000). This

procedure was recently validated and proposed as the international standard diagnostic method for quality assurance laboratories in epidemiological studies on *Salmonella* spp. (Malorny, *et al.*, 2003). The PCR was carried out in a total volume of 50 µl containing 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl *Taq* polymerase (5 U μl^{-1}), and 1 µl of each primer (100 ng μl^{-1}) and 1 µl of the cell lysates (Hahn, *et al.*, 2007). The PCR was performed in a PTC-200 thermocycler (MJ Research, Waltham, MA) with an initial denaturation at 96ºC for 2 minutes, followed by 35 rounds of temperature cycling with denaturation at 96ºC, primer annealing at 64ºC, elongation at 72ºC, each for 30 seconds (Malorny, *et al.*, 2003). *Salmonella typhimurium* ATCC 14028 was used as a positive control. PCR products were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 µg ml^{-1}) (Sambrook, *et al.*, 1989).

Isolation and Rep-PCR analyses

Sub-samples (100 µl) of the second enrichment were plated on RVS agar (RVS solidified with 15 g agar L^{-1}) and incubated for 16 hours. From each sample, 10 to 40 colonies were chosen haphazardly and incubated in Luria–Bertani broth (LB; L^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) at 37ºC for 7 hours. Cells from 100-µl sub-samples were pelleted by centrifugation, and lysed in 100 µl 50 mM NaOH as described above. Isolates representing salmonellae were identified by PCR targeting the *inv*A gene as described above, and further characterized by rep-PCR, a

PCR-assisted fingerprinting technique targeting consensus motifs of repetitive elements common to prokaryotic genomes (Bennasar, *et al.*, 2000, Woo & Lee, 2006). Rep-PCR was performed in a total volume of 25 µl with primer BoxA1R ($^{5'}$CTA CGG CAA GGC GAC GCT GAC G), and 2 µl of lysate as described in (Hahn, *et al.*, 2007). Banding profiles were screened visually by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook, *et al.*, 1989), and representative profiles documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA).

Results

After semi-selective enrichment in RVS, *inv*A gene fragments were detected by PCR in biofilm samples only, while water samples remained always negative independent of location and sampling time. The two sampling sites provided different overall detection results. For the Spring Lake site, enrichments of the 20 biofilm sampling locations within the 324 cm^2-area resulted in low initial *inv*A gene fragment detection, with only one out of 20 locations being positive for the April 24 and May samples, and no detection at all for the May 20 samples (Table 2.1). Enrichments from biofilms collected September 21 and 25 displayed a much higher detection rate, with 8 locations each being positive for *inv*A gene fragments (Table 2.2). For all 5 sampling times combined, individual locations were generally positive only once or twice (both 6 out of 20 locations within the 324 cm^2 sampling area) for a total of 18% of all samples, while 8 locations were always negative (Fig. 2.3).

Table 2.1. Distribution and diversity of salmonellae in biofilms collected from sites Spring Lake and City Park four times during Spring 2009[1]

Sample location[2]	No. of colonies checked per location[3]	No. of colonies identified as salmonellae (in % of all checked)	Rep-PCR profiles of colonies identified as salmonellae	
			S12	S24
Spring Lake				
April 24				
A1	20	19 (95%)	**19**	
May 5				
E1	40	21 (57%)	**8**	**13**
May 20				
none	40	0		
City Park				
June 2				
B3	10	10 (100%)	**10**	
C1	10	9 (90%)	**9**	

[1]samples were collected from a small area (12 x 27 cm) in a sampling grid that consisted of 4 rows (1 – 4) and 5 columns (A – E) for a total of 20 locations (see Figure 3)

[2]locations not shown were negative for salmonellae

[3]enrichments of all 20 locations were tested but none of those negative for the *inv*A gene resulted in the isolation of any salmonellae

Table 2.2 Distribution and diversity of salmonellae in biofilms collected from site City Park June 22, 2009[1]

Sample[2] (replicate)	No. of colonies identified as salmonellae (in % of all checked)[3]	Rep-PCR profiles of colonies identified as salmonellae					
		S25	S26	S27	S28	S29	S30
A3 (1)	5 (50%)		**4**		**1**		
(2)	0						
(3)	0						
A4 (1)	0						
(2)	8 (80%)	**2**	**4**	**2**			
(3)	5 (50%)		**5**				
B1 (1)	10 (100%)		**10**				
(2)	0						
(3)	2 (20%)				**1**	**1**	
B3 (1)	1 (10%)	**1**					
(2)	6 (60%)	**2**	**4**				

Table 2.2 Cont.

(3)	6 (60%)		**6**			
C1 (1)	0					
(2)	0					
(3)	10 (100%)		**7**			**3**
C2 (1)	0					
(2)	10 (100%)		**9**	**1**		
(3)	2 20%)	**1**	**1**			
C3 (1)	3 (30%)		**3**			
(2)	7 (70%)		**7**			
(3)	9 (90%)		**9**			
C4 (1)	4 (40%)		**3**	**1**		
(2)	0					
(3)	3 (30%)		**3**			
D1 (1)	0					
(2)	0					
(3)	10 (100%)		**10**			
E1 (1)	3 (30%)		**3**			
(2)	3 (30%)	**1**	**2**			
(3)	0					

[1]samples were collected from a small area (12 x 27 cm) in a sampling grid that consisted of 4 rows (1 – 4) and 5 columns (A – E) for a total of 20 locations (see Figure 3)

[2]locations not shown were negative for salmonellae

[3]enrichments of all 20 locations were tested but none of those negative for the *inv*A gene resulted in the isolation of any salmonellae (n=10 colonies tested)

For the City Park sampling area, enrichment for all 20 locations displayed the presence of *inv*A gene fragments at least once, with most locations generally being positive twice (11 out of 20 locations) or 3 times (6 locations) out of 4 sampling times for a total of 59% positive samples (Fig. 2.3). Similar to Spring Lake samples, enrichments of biofilm samples collected in spring (i.e., June 2) revealed the presence

of *inv*A gene fragments at only few (i.e., 2) locations (Table 2.1), while *inv*A gene fragments were detected in enrichments of many more biofilm samples collected in June 22, September 21 and September 25, with 10, 16 and 17 of the 20 locations, respectively, being positive (Table 2.3, 2.4).

Table 2.3 Distribution and diversity of salmonellae in biofilms collected from site Spring Lake September 21 and 25, 2009[1]

Sample[2]	No. of colonies identified as salmonellae (in % of all checked)[3]	Rep-PCR profiles of colonies identified as salmonellae						
		S1	S2	S3	S4	S12	S17	S24
September 21								
A3	1 (10%)	**1**						
A4	10 (100%)	**8**				**2**		
B2	8 (80%)		**1**	**5**	**1**	**1**		
C1	9 (90%)	**9**						
C3	10 (100%)	**10**						
D1	1 (10%)	**1**						
E1	9 (90%)	**9**						
E3	10 (100%)	**10**						
September 25								
A1	6 (60%)	**6**						
A3	6 (60%)	**3**				**3**		
A4	8 (80%)					**8**		
C2	5 (50%)		**1**		**1**	**2**	**1**	
C3	7 (70%)	**7**						
D2	4 (40%)	**4**						
D3	8 (80%)	**7**				**1**		
E3	4 (40%)					**4**		

[1]samples were collected from a small area (12 x 27 cm) in a sampling grid that consisted of 4 rows (1 – 4) and 5 columns (A – E) for a total of 20 locations (see Figure 3)

[2]locations not shown were negative for salmonellae

[3]enrichments of all 20 locations were tested but none of those negative for the *inv*A gene resulted in the isolation of any salmonellae (n=10 colonies tested)

Table 2.4 Distribution and diversity of salmonellae in biofilms collected from site City Park September 21 and 25, 2009[1]

Sample[2]	No. of colonies identified as salmonellae (in % of all checked)[3]	Rep-PCR profiles of colonies identified as *Salmonella*																			
		S1	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S18	S19	S20	S21	S22	S23
September 21																					
A1	90										9										
A2	90				6						1			2							
A3	90				1					6	2										
A4	70				6										1						
B2	100				9					1											
B3	20				2																
B4	100									6	3		1								
C1	100				8					2											
C2	80				8																
C3	100									9	1										
C4	60									6											
D1	90					1					3	5									
D2	70						5	2													
D3	90			7						2											
D4	20			2																	
E2	70	5							1		1										
September 25																					
A2	10																	1			
A4	30									1								2			
B1	20									1								1			
B2	40													2				1			1
B3	30													1					1		1
B4	30																	2		1	
C2	40													1					3		
C3	40										4										
C4	70										2								5		
D1	80										2								6		
D2	80		1															1	6		
D3	70																7				
D4	30				3																
E1	60	4									2										
E2	50	3									1					1					
E3	20	2																			
E4	90	9																			

[1]samples were collected from a small area (12 x 27 cm) in a sampling grid that consisted of 4 rows (1 – 4) and 5 columns (A – E) for a total of 20 locations; [2]locations not shown were negative for salmonellae; [3]enrichments of all 20 locations were tested but none of those negative for the *inv*A gene resulted in the isolation of any salmonellae (n=10 colonies tested)

Isolation of salmonellae was achieved only from enrichments of biofilm samples that had tested positive for the presence of *inv*A gene fragments, but not from those being negative including enrichments of water samples. The number of isolates obtained was highly variable, with numbers covering the range of one to all colonies checked being positive for the *inv*A gene (Tables 2.1-4). Overall, 491 isolates obtained from both City Park and Spring Lake samples were identified as *Salmonella* by the

presence of the *inv*A gene. Rep-PCR fingerprinting of these isolates resulted in 30 distinct profiles (Fig. 2.4), with 26 and 7 profiles retrieved from City Park and Spring Lake samples, respectively (Tables 2.1-4). Three isolates with identical profiles (S1, S4, and S12) were present at both sites.

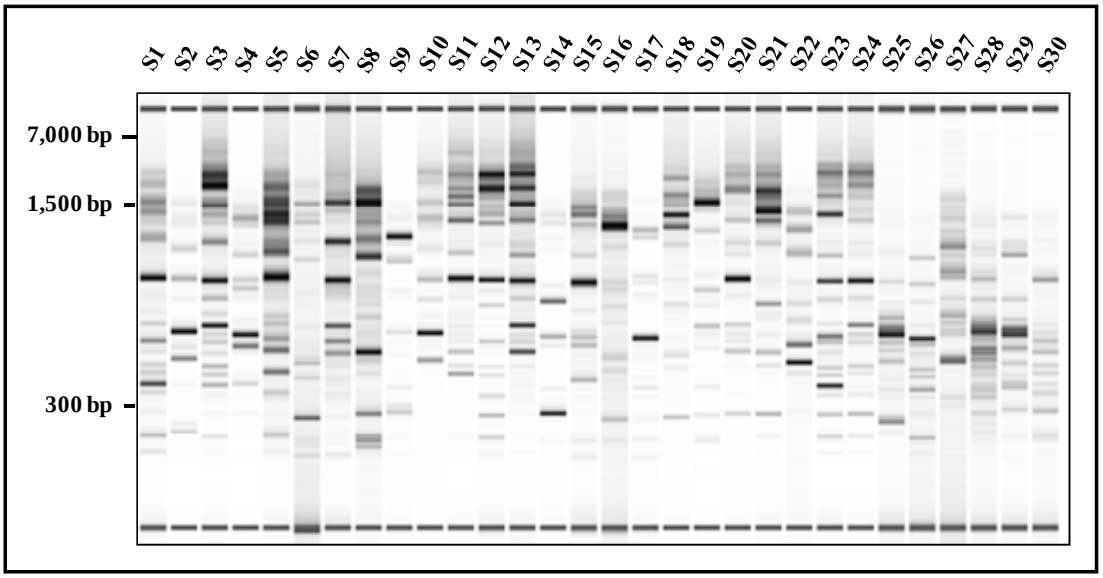


Fig. 2.4 Representative rep-PCR profiles (S1 – S30) of isolates from enrichment cultures for salmonellae from biofilm samples from both Spring Lake and City Park, documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA). Fragment sizes on the left represent those determined by the Bioanalyzer.

Multiple strains (i.e., represented by up to 4 rep-PCR profiles) were frequently obtained from single locations at both sites (Table 2.1-4), as well as from replicate enrichments from the same biofilms (Table 2.3). Replicate enrichments varied with respect to the detection of *inv*A gene fragments from 1 to all 3 replicates being positive, as well as with respect to diversity with isolates from replicates being represented by different rep-PCR profiles (Table 2.3). The composition of *Salmonella*

strains in the entire 324 cm^2 area analyzed changed in time with large differences between early (April, June) and late sampling times (September) within and among sites City Park and Spring Lake (Tables 1-4). Only one strain (S12) was abundant at almost all sampling times at both sites, though often at different locations within the area analyzed.

Discussion

PCR detected salmonellae after semi-selective enrichment of biofilm samples in a patchy distribution in the 324 cm^2 sampling area at both sites, with overall lower detection frequency and diversity in samples from Spring Lake compared to samples from City Park. Though both sites are located in areas characterized as grass- and parkland with abundant wildlife that include large numbers of deer or other animals that have been shown to host salmonellae (Bigler, *et al.*, 1974, Refsum, *et al.*, 2002, Briones, *et al.*, 2004, Branham, *et al.*, 2005, Renter, *et al.*, 2006), the differences in detection frequency and diversity of salmonellae between sites are most likely a consequence of specific environmental characteristics. Spring Lake is generally considered one of the most pristine waters in Texas (Slattery & Fahlquist, 1997) fed by a system of 200 artesian springs of the Edwards Aquifer with an average cumulative discharge of approximately 4.8 m^3 per second (Slattery & Fahlquist, 1997). The sampling site was located just upstream of these springs which excludes potential contamination from upstream water. It is surrounded by concrete walkways and buildings, with few food resources for animals, restricting the size of the potential

contamination area and minimizing the accumulation of fecal droppings from wildlife in this area (Fig. 1). Thus large rainfall events, in addition to potential small scale contamination by animals, are necessary to produce runoff significant enough to occasionally wash animal droppings into the system with a short residence time due to the fast water flow. This is different for the City Park site where a permanent slow flow of water from the upstream ponds that cover a much larger area with adequate food resources for animals than the Spring Lake site, might result in much longer exposure to contaminating feces and thus salmonellae (Fig. 2.1). Biofilms at the Spring Lake site might therefore only be exposed to contaminating runoff for a short time, while exposure of biofilms at the City Park site is longer and, as a consequence of the larger area contributing to contamination, populations of salmonellae more diverse.

Patchiness in the detection of salmonellae was not only observed in the 324 cm^2 area, but also on smaller scale. This was evident for replicate samples that demonstrated large differences in the detection of salmonellae within a single 3 cm^2 sampling area of most locations (Table 2.3). This patchiness is likely the consequence of a non-homogeneous distribution of salmonellae in our original sample due to the binding of salmonellae to particulate material or components of the biofilm, exacerbated by insufficient release during our homogenization attempts. Biofilms are highly heterogeneous communities of different microorganisms including diatoms, green algae, protozoa, fungi and bacteria that represent hot spots of rapidly available

carbon resources for heterotrophic organisms (Geesey, *et al.*, 1978, Augspurger, *et al.*, 2008). Biofilms might therefore provide habitat suitable not only for long term survival of salmonellae in aquatic systems, but could actually provide the opportunity for growth as suggested for other non-enteric environments such as soil (Topp, *et al.*, 2003, You, *et al.*, 2006). While our study was not designed to assess growth of salmonellae in natural biofilms, the detection of salmonellae confirms that salmonellae can persist in biofilms for some time and supports previous conclusions for the survival of salmonellae in algal mats (Ishii, *et al.*, 2006, Englebert, 2008, Byappanahalli, *et al.*, 2009).

The results also demonstrated a significant micro-heterogeneity of *Salmonella* strains, detecting up to 13 different strains in the 324 cm^2 sampling area and up to 4 different strains at one location (3 cm^2), as indicated by rep-PCR. Rep-PCR is a high resolution tool with the ability to differentiate closely related microbial strains (Hyytiä-Trees, *et al.*, 2007, Foley, *et al.*, 2009). It allows discrimination among closely related strains of *Salmonella* (Albufera, *et al.*, 2009, Ben-Darif, *et al.*, 2010), with potentially better resolution than obtained by traditional serological assays or sequence analyses of inter-spacer regions of the *rrf*H gene (Wise, *et al.*, 2009). Consequent of its high sensitivity in discriminating among *Salmonella*, its application has recently been suggested as an alternative to traditional serotyping methodologies (Anderson, *et al.*, 2010). Rep-PCR therefore provides sensitive information essential for bacterial source tracking and to determine the distribution of this pathogen in general or of specific

strains in particular. Our results also support prior conclusions that distribution and overall diversity of salmonellae might easily be underestimated in large scale or seasonal sampling schemes as well as from the analyses of limited numbers of samples or isolates (Ishii, *et al.*, 2006, Byappanahalli, *et al.*, 2009).

Studies on the long-term persistence, or seasonal variation on presence or diversity of salmonellae, however, were impacted by the destructive sampling that is required in all microbial ecology studies, and thus, despite attempts to re-sample the same site or a locale very close to the original sampling site, such replicates might not retrieve the same strains or microdiversity in time, e.g. before and after precipitation related runoff. Studies on the establishment of a dominant and potentially environmental strain such as S12 that was present at both sites at almost all sampling times, however, and potential changes through time should be feasible and provide additional detail on the long-term persistence or growth of salmonellae in biofilms. Such studies, however, will require technical modifications that seek to refine accurate sample retrieval and take into consideration additional quantitative analyses.

Acknowledgements

The authors are grateful for financial support from Texas State University-San Marcos, Department of Biology, and the State of Texas through the American Recovery and Reinvestment Act (ARRA).

References

[1] Albufera U, Bhugaloo-Vial P, Issack MI & Jaufeerally-Fakim Y (2009) Molecular characterization of *Salmonella* isolates by REP-PCR and RAPD analysis. *Infection, Genetics and Evolution* **9**: 322-327.

[2] Anderson PN, Hume ME, Byrd JA, Hernandez C, Stevens SM, Stringfellow K & Caldwell DJ (2010) Evaluation of repetitive extragenic palindromic-polymerase chain reaction and denatured gradient gel electrophoresis in identifying *Salmonella* serotypes isolated from processed turkey. *Poultry Science* **89**: 1293-1300.

[3] Augspurger C, Gleixner G, Kramer C & Küsel K (2008) Tracking carbon flow in a 2-week-old and 6-week-old stream biofilm food web. *Limnology and Oceanography* **53**: 642-650.

[4] Baudart J, Grabulos J, Barusseau JP & Lebaron P (2000) *Salmonella* spp. and fecal coliform loads in coastal waters from a point vs. nonpoint source of pollution. *Journal of Environmental Quality* **29**: 241-250.

[5] Ben-Darif E, De Pinna E, Threlfall EJ, Bolton FJ, Upton M & Fox AJ (2010) Comparison of a semi-automated rep-PCR system and multilocus sequence typing for differentiation of *Salmonella enterica* isolates. *Journal of Microbiological Methods* **81**: 11-16.

[6] Bennasar A, de Luna G, Cabrer B & Lalucat J (2000) Rapid identification of *Salmonella typhimurium, S. enteritidis* and *S. virchow* isolates by Polymerase Chain Reaction based fingerprinting methods. *International Microbiology* **3**: 31-38.

[7] Bigler WJ, Hoff GL, Jasmin AM & White FH (1974) *Salmonella* infections in Florida raccoons, *Procyon loto*. *Archives of Environmental Health* **28**: 261-262.

[8] Branham LA, Carr MA, Scott CB & Callaway TR (2005) *E. coli* O157 and *Salmonella* spp. in White-tailed deer and livestock. *Current Issues in Intestinal Microbiology* **6**: 25-29.

[9] Briones V, Téllez S, Goyache J, Ballesteros C, del Pilar Lanzarot M, Domínguez L & Fernández-Garayzábal JF (2004) *Salmonella* diversity associated with wild reptiles and amphibians in Spain. *Environmental Microbiology* **6**: 868-871.

[10] Byappanahalli MN, Sawdey R, Ishii S, Shively DA, Ferguson JA, Whitman RL & Sadowsky MJ (2009) Seasonal stability of *Cladophora*-associated *Salmonella* in Lake Michigan watersheds. *Water Resources* **43**: 806-808.

[11] Chandran A & Hatha AAM (2005) Relative survival of *Escherichia coli* and *Salmonella typhimurium* in a tropical estuary. *Water Resources* **39**: 1397-1403.

[12] Chao W, Ding R & Chen R (1987) Survival of pathogenic bacteria in environmental microcosms. *Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi* **20**: 339-348.

[13] Cote C & Quessy S (2005) Persistence of *Escherichia coli* and *Salmonella* in surface soil following application of liquid hog manure for production of pickling cucumbers. *Journal of Food Protection* **68**: 900-905.

[14] Englebert ET, McDermott C & Kleinheinz GT (2008) Impact of the alga *Cladophora* on the survival of *E. coli*, *Salmonella*, and *Shigella* in laboratory microcosm. *Journal of Great Lakes Research* **34**: 377-382.

[15] Fair JF & Morrison SM (1967) Recovery of bacterial pathogens from high quality surface waters. *Water Resources Research* **3**: 799-803.

[16] Foley SL, Lynne AM & Nayak R (2009) Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. *Infection, Genetics and Evolution* **9**: 430-440.

[17] Foltz VD (1969) *Salmonella* Ecology. *Journal of the American Oil Chemists' Society* **46**: 222-224.

[18] Gaertner JP, Forstner MRJ, Rose FL & Hahn D (2008) Detection of salmonellae in different turtle species within a headwater spring ecosystem. *Journal of Wildlife Diseases* **44**: 519-526.

[19] Gaertner JP, Garres T, Becker JC, Jimenez ML, Forstner MJR & Hahn D (2009) Temporal analyses of salmonellae in a headwater spring ecosystem. *Journal of Water and Health* **7**: 115-12.

[20] Geesey GG, Mutch R, Costerton JW & Green RB (1978) Sessile bacteria - important component of microbial population in small mountain streams. *Limnology and Oceanography* **23**: 1214-1223.

[21] Gray LD (1995) *Escherichia, Salmonella, Shigella,* and *Yersinia*. In: P.R. Murray (ed.), Manual of clinical microbiology. ASM Press, Washington, DC, pp. 450-456.

[22] Hahn D, Gaertner J, Forstner MJR & Rose FL (2007) High resolution analysis of salmonellae from turtles within a headwater spring ecosystem. *FEMS Microbiology Ecology* **60**: 148-155.

[23] Haley BJ, Cole DJ, E.K. Lipp (2009) Distribution, diversity, and seasonality of waterborne salmonellae in a rural watershed. *Applied Environmental Microbiology* **75**: 1248-1255.

[24] Harvey RWS, Price TH, Foster DW, Griffith WC (1969) *Salmonellas* in sewage. A study in latent human infection. *Journal of Hygiene* **67**: 517-523.

[25] Hendricks CW (1971) Increased recovery rate of salmonellae from stream bottom sediments versus surface waters. *Applied and Environmental Microbiology* **21**: 379-380.

[26] Hendricks CW & Morrison SM (1967) Multiplication and growth of selected enteric bacteria in clear mountain stream water. *Water Resources* **1**: 567-576.

[27] Humphrey T (2000) Public-health aspects of *Salmonella* infection, CABI Publishing, Wallingford, UK.

[28] Hyytiä-Trees EK, Cooper K, Ribot EM & Gerner-Smidt P (2007) Recent developments and future prospects in subtyping of foodborne bacterial pathogens. *Future Microbiology* **2**: 175-185.

[29] International Standard Organization (1993) Detection of salmonellae (reference method). International Standard ISO 6579.

[30] Ishii S, Yan T, Shively DA, Byappanahalli MN, Whitman RL & Sadowsky MJ (2006) *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. *Applied and Environmental Microbiology* **72**: 4545-4553.

[31] Islam M, Morgan J, Doyle MP, Phatak SC, Millner PD & Jiang X (2004) Persistence of *Salmonella enterica* serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fileds treated with contaminated manure composts or irrigation water. *Foodborne Pathogens and Diseases* **1**: 27-35.

[32] Khan AA, Navaz MS, Khan SA & Cerniglia CE (2000) Detection of multidruge-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. *FEMS Microbiology Letters* **182**: 355-360.

[33] Malorny B, Hoorfar J, Bunge C & Helmuth R (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: Towards an international standard. *Applied and Environmental Microbiology* **69**: 290-296.

[34] Martinez-Urtaza J, Saco M, de Novoa J, Perez-Pineiro P, Peiteado J, Lozano-Leon A & Garcia-Martin O (2004) Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. *Applied and Environmental Microbiology* **70**: 2089-2097.

[35] Nakadai A, Kuroki T, Kato Y, Suzuki R, Yamai S, Yaginuma C, Shiotani R, Yamanouchi A & Hayashidani H (2005) Prevalence of *Salmonella* spp. in pet reptiles in Japan. *The Journal of Veterinary Medical Science* **67**: 97-101.

[36] World Health Organization (2002) World Health Report, Reducing Risks, Promoting Healthy Life. World Health Organization.

[37] Polo F, Figueras MJ, Inza I, Sala J, Fleisher JM & Guarro J (1998) Relationship between presence of *Salmonella* and indicators of fecal pollution in aquatic habitats. *FEMS Microbiology Letters* **160**: 253-256.

[38] Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galán JE, Ginocchio C, Curtiss R & Gyles CL (1992) Amplification of an *inv*A gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes* **6**: 271-279.

[39] Refsum T, Heir E, Kapperud G, Vardund T & Holstad G (2002) Molecular epidemiology of *Salmonella enterica* serovar Typhimurium isolates determined by pulsed-field gel electrophoresis: Comparison of isolates from avian wildlife, domestic animals and the environment in Norway. *Applied and Environmental Microbiology* **68**: 5600-5606.

[40] Renter DG, Gnad DP, Sargeant JM & Hygnstrom SE (2006) Prevalence and serovars of *Salmonella* in the feces of free-ranging White-tailed deer (*Odocoileus virginianus*) in Nebraska. *Journal of Wildlife Diseases* **42**: 699-703.

[41] Sambrook J, Fritsch EF & Maniatis T (1989) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[42] Sanyal D, Douglas T & Roberts R (1997) *Salmonella* infection acquired from reptilian pets. *Archives of Disease in Childhood* **77**: 345-346.

[43] Semenov AV, van Overbeek L & van Bruggen AH (2009) Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. *Applied and Environmental Microbiology* **75**: 3206-3215.

[44] Slattery RN & Fahlquist L (1997) Water quality summary of the San Marcos springs riverine system, San Marcos, Texas, July-August 1994. USGS Survey FS-059-57, San Antonio, TX.

[45] Suárez M & Rüssmann H (1998) Molecular mechanisms of *Salmonella* invasion: The type III secretion system of the pathogenicity island 1. *International Microbiology* **1**: 197-204.

[46] Tauxe RV (1997) Emerging foodborne diseases: An evolving public health challenge. *Emerging Infectious Diseases* **3**: 425-434.

[47] Thomason BM, Biddle JW & Cherry WB (1975) Detection of Salmonellae in the environment. *Applied Microbiology* **30**: 764-767.

[48] Topp E, Welsh M, Tien YC, Dang A, Lazarovits G, Conn K & Zhu H (2003) Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. *FEMS Microbiology Ecology* **44**: 303-308.

[49] Turnbull PCB (1979) Food poisoning with special reference to *Salmonella* - Its epidemiology, pathogenesis and control. *Clinics in Gastroenterology* **8**: 663-714.

[50] Turpin PE, Maycroft KA, Rowlands CL & Wellington EMH (1993) Viable but not-culturable Salmonellas in soil. *The Journal of Applied Bacteriology* **74**: 421-427.

[51] Vassiliadis P, Kalapothaki V, Trichopoulos D, Mavrommatti C & Serie C (1981) Improved Isolation of Salmonellae from Naturally Contaminated Meat Products by Using Rappaport-Vassiliadis Enrichment Broth. *Applied and Environmental Microbiology* **42**: 615-618.

[52] Watnick PI & Kolter R (1999) Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Molecular Microbiology* **34**: 586-595.

[53] Wells EVM, Boulton M, Hall W & Bidol SA (2004) Reptile-associated salmonellosis in preschool-aged children in Michigan, January 2001-June 2003. *Clinical Infectious Diseases* **39**: 687-691.

[54] Wise MG, Siragusa GR, Plumblee J, Healy M, Cray PJ & Seal BS (2009) Predicting *Salmonella enterica* serotypes by repetitive sequence-based PCR. *Journal of Microbiological Methods* **76**: 18-24.

[55] Woo YK & Lee SH (2006) Genetic diversity of multi-resistant *Salmonella enterica* serotype Typhimurium isolates from animals and humans. *Journal of Microbiology* **44**: 106-112.

[56] Yildiz FH & Schoolnik GK (1999) *Vibrio cholerae* O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 4028-4033.

[57] You Y, Rankin SC, Aceto HW, Benson CE, Toth JD & Dou Z (2006) Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Applied and Environmental Microbiology* **72**: 5777-5783.

CHAPTER 3

QUANTIFYING *SALMONELLA* POPULATION DYNAMICS IN WATER AND BIOFILMS

Sha Q, Vattem DA, Forstner MRJ & Hahn D. 2013. Quantifying *Salmonella* population dynamics in water and biofilms. *Microb. Ecol.* **(in press)**.

Abstract

Members of the bacterial genus *Salmonella* are recognized worldwide as major zoonotic pathogens that are often found to persist in non-enteric environments including heterogeneous aquatic biofilms. In this study, *Salmonella* isolates that had been detected repeatedly over time in aquatic biofilms at different sites in Spring Lake, San Marcos, TX, were identified as serovars Give, Thompson, Newport and -:z10:z39. Pathogenicity results from feeding studies with the nematode *Caenorhabditis elegans* as host confirmed that these strains were pathogenic, with *Salmonella*-fed *C. elegans* dying faster (mean survival time between 3 and 4 days) than controls, i.e. *Escherichia coli*-fed *C. elegans* (mean survival time of 9.5 days). Cells of these isolates inoculated into water at a density of up to 10^6 ml^{-1} water declined numerically by 3-orders of magnitude within 2 days, reaching the detection limit of our *q*PCR-based quantification technique (i.e. 10^3 cells ml^{-1}). Similar patterns were obtained for cells in heterogeneous aquatic biofilms developed on tiles and originally free of *Salmonella* that were kept in the inoculated water. Cell numbers increased during the first days to more than 10^7 cells cm^{-2}, and then declined over time. Ten-fold higher cell numbers of *Salmonella* inoculated into water or into biofilm resulted in similar patterns of population dynamics, though cells in biofilms remained detectable with numbers around 10^4 cells cm^{-2} after 4 weeks. Independent of detectability by *q*PCR, samples of all treatments harbored viable salmonellae that resembled the inoculated isolates after 4 weeks of incubation. These results demonstrate that pathogenic salmonellae were

isolated from heterogeneous aquatic biofilms and that they could persist and stay viable in such biofilms in high numbers for some time.

Introduction

Members of the bacterial genus *Salmonella* are recognized worldwide as major zoonotic pathogens, responsible for an estimated 93.8 million cases of gastroenteritis and 155,000 deaths in humans annually (Humphrey, 2000, Hoelzer, *et al.*, 2011). Although direct contact to animals carrying salmonellae has been identified as an avenue for infection (Sanyal, *et al.*, 1997, Wells, *et al.*, 2004), salmonellae are typically transmitted to humans via food and drinking water contaminated with feces of animals (Tauxe, 1997, World Health Organization, 2002). The intestinal tract of vertebrates is presumed to be the native habitat of salmonellae (Woodward, *et al.*, 1997), despite *Salmonella* sp. being frequently detected in non-enteric environments such as water (Cherry, *et al.*, 1972, Cherry, *et al.*, 1975, Jiménez, *et al.*, 1989, Martinez-Urtaza, *et al.*, 2004), soils and sediments (Cote & Quessy, 2005, Danyluk, *et al.*, 2008), as well as algae and biofilms (Ishii, *et al.*, 2006, Gaertner, *et al.*, 2008, Byappanahalli, *et al.*, 2009, Gaertner, *et al.*, 2011, Sha, *et al.*, 2011).

Algae and biofilms provide habitats suitable for survival of enteric pathogens such as *Escherichia coli* (Domingo, *et al.*, 1989, Ishii, *et al.*, 2006, Semenov, *et al.*, 2009) or *Salmonella* (Byappanahalli, *et al.*, 2003, Ishii, *et al.*, 2006, Ksoll, *et al.*, 2007, Byappanahalli, *et al.*, 2009). Therefore, they represent environments for potential long-term survival of these pathogens in aquatic systems, as discussed for other

organisms (Watnick & Kolter, 1999, Yildiz & Schoolnik, 1999), or even growth as suggested for other environments such as soil (Topp, *et al.*, 2003, You, *et al.*, 2006). Similar to animal carriers, algae and biofilms might therefore serve as reservoirs for water contamination, effectively increasing the infective dose of pathogens in the environment after release from biofilms and thus increasing the incidence of disease in humans with contact to contaminated water (Purevdorj, 2002, Marsollier, *et al.*, 2004).

We have recently demonstrated the presence of salmonellae in natural biofilms on concrete surfaces and a significant micro-heterogeneity with differences in diversity of viable salmonellae at 2 sites in San Marcos, Texas, i.e., Spring Lake, the spring-fed headwaters of the San Marcos river, and City Park, a stormwater overflow channel (Sha, *et al.*, 2011). Several isolates were found at both sites and at different sampling times during the season suggesting either long-term persistence outside potential animal hosts or iterative re-inoculation through feces of carriers. In order to evaluate the potential of these isolates to persist or even grow in water and biofilms, four isolates were initially characterized with respect to serotype and for pathogenicity to assess their potential threat to human health, and then used to inoculate either water or heterogeneous aquatic biofilms in aquaria mesocosms. Biofilms had been grown on tiles with defined surface area in a spring-fed stream channel before placement into the aquaria and were originally free of salmonellae. Population dynamics of salmonellae in both water and biofilm samples were followed over a 4-week period

using quantitative PCR (*q*PCR). At the end of that period, attempts were made to enrich for and isolate viable salmonellae from both environments.

Material and Methods

Selection and characterization of *Salmonella* isolates Initial studies focused on isolates from biofilms obtained in a recent study (Sha*, et al.*, 2011): isolate S12 was detected at four sampling times in spring and fall at two sites adjacent but not connected, Spring Lake and City Park; S11 was isolated at two sampling times in fall, though in biofilms from City Park only; S3 was only detected once in biofilms from Spring Lake, and S19 only once in biofilms from City Park. These strains were confirmed as being salmonellae and serotyped using a combination of agglutination assays and PCR-based assays (molecular serotyping) at the Texas Department of State Health Services (Austin, TX) that also characterized them by pulsed-field gel electrophoresis (PFGE) after DNA cleavage with XbaI.

Nematode-killing assay for pathogenecity All isolates as well as *Salmonella enterica* serovar Typhimurium LT2 (ATCC 19585) were tested for pathogenicity using feeding studies with *Caenorhabditis elegans* (Aballay*, et al.*, 2000, Labrousse*, et al.*, 2000, Zachow*, et al.*, 2009). Nematodes (*C. elegans* strain Bristol N2) were kept as hermaphrodites on nematode growth medium (NGM) agar (L^{-1}: 2.5 g peptone, 3 g NaCl, 17 g agar, 1 ml of 1 M cholesterol, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, 1 ml of 1 M potassium phosphate buffer, pH 6) at 20℃ (Brenner, 1974) and fed with *Escherichia coli* strain OP 50. For each strain (i.e. isolates S11, S12, S3 and S19, *S.*

enterica serovar Typhimurium LT2, and *E. coli* OP 50), pathogenicity assays were conducted on NGM agar plates (60 x 15 mm). Plates (n=6 per strain) were inoculated with 25 µl of bacterial culture that was grown in Luria–Bertani broth (LB) (L^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) at 37℃ for 10 hrs, and was adjusted to an OD_{564} = 1.03 ± 0.02. After incubation at 37℃ for 10 hrs, each plate received 10 to 15 individuals of *C. elegans* that were 3-days of age. After incubation at room temperature for 24 hrs, living *C. elegans* from each plate were transferred onto a plate that contained *E. coli* OP 50 that had been grown at 37℃ for 10 hrs. *C. elegans* were observed under a Leica EZ4 dissecting microscope (Leica Microsystems Inc., Buffalo Grove, IL) after 24 hrs, and living individuals transferred to fresh plates with *E. coli* OP 50. These transfers were repeated daily for a total of 11 days.

For statistical purposes three replicates per experiment with a total of 90 nematodes were used. Failure to respond to touch and the absence of pharyngeal pumping was used to score dead individuals. The Kaplan-Meier method was used to compare the survival curves. Survival differences were tested for significance ($p<0.001$) using the Gehan-Breslow-Wilcoxon test in GraphPad Prism, version 5.0 (GraphPad Software, Inc., San Diego, CA).

Growth studies of salmonellae in biofilms and water For growth studies under controlled conditions, heterogeneous aquatic biofilms were grown on ceramic tiles (2.2 x 2.2 cm, non-glazed) in a stream channel adjacent to the Freeman Aquatic Building at Texas State University-San Marcos with running spring water for 3

months. Biofilms on tiles were assumed to be free of salmonellae when PCR-based detection attempts, i.e. PCR after semi-selective enrichment in Rappaport-Vassiliadis Broth (RVS) broth (Gaertner*, et al.*, 2009, Sha*, et al.*, 2011), and *q*PCR on DNA extracts from these biofilms (see below) remained negative. Tiles with biofilms were then used for growth studies performed in 36 L-aquaria in the laboratory. In all experiments, tiles with biofilms were covered with 10 L of dechlorinated tap water resulting in a water level of about 6.5 cm in the aquaria, and incubated at room temperature (i.e. 25℃) and artificial light conditions at a 16/8 day/night photoperiod for 4 weeks.

Isolates S11, S12, S3 and S19 previously collected from aquatic biofilms and *S. enterica* serovar Typhimurium LT2 were grown in LB medium for 16 hrs, washed with tap water twice, and inoculated into the water covering the biofilms at a density of approximately 10^6 cells ml^{-1} estimated from the OD_{564} reading (3 aquaria for each strain, with 54 tiles each). Samples were collected immediately after inoculation, after 12, 24, 36 and 48 hours, and then 3, 4, 5, 6 and 7 days, and finally 2, 3 and 4 weeks later. At each time, three tiles were collected from each aquarium with a pair of sterilized forceps, rinsed with sterilized distilled water and transferred to a 50-ml tube where they were covered with 10 ml of sterilized distilled water. Biofilms were released from tiles by sonication for 5 minutes (sonic cleaner 2QT; Fisher Scientific Inc., Pittsburgh, PA), after which tiles were removed and cells collected by centrifugation at 3,200 x g for 15 minutes. Concurrently, 40 ml of water were

removed with sterile 60-ml syringes, transferred to sterile 50-ml tubes and centrifuged at 3,200 x g for 15 minutes. Cell pellets from biofilm and water samples were then re-suspended in 100 or 50 µl of 50 mM NaOH, respectively. Additional samples were taken after 4 weeks of incubation and analyzed to confirm the presence of the inoculated strain. This analysis used isolates obtained after semi-selective enrichment of salmonellae and characterization by rep-PCR as described previously (Sha*, et al.*, 2011).

These analyses were repeated with an isolate obtained from sediments of the slough arm of Spring Lake that was characterized as *Salmonella enterica* serovar Newport by the Texas Department of State Health Services (Austin, TX) (Gaertner*, et al.*, 2009). In the initial setup, this strain was also inoculated into the water covering biofilms, though at an estimated density of 10^7 cells ml^{-1} in 6 aquaria, with 54 tiles each. Tiles from 3 aquaria were transferred 1 hour later to 3 clean (i.e. *Salmonella*-free) aquaria, and were covered subsequently with 10 L of tap water (referred to as treatment 3, clean water, inoculated biofilm). In addition to the initial setup (treatment 2, inoculated water, clean biofilm), a set of 3 aquaria without biofilm, but inoculated water was used as treatment 1, and 3 aquaria with clean biofilm and clean water were used as control.

Quantification of salmonellae in biofilms and water by *q*PCR Resuspended cells from biofilm and water samples were lysed at 65℃ for 30 min (Sha*, et al.*, 2011). While lysates from water samples were directly used as template in *q*PCR analyses,

DNA from biofilm samples was purified using the UltraClean™ 15 DNA purification kit (MoBio, Carlsbad. CA). Extraction efficiencies were determined by *q*PCR quantification of added DNA of the nitrogen-fixing symbiont *Frankia* (strain Ag45/Mut15) before and after purification (Samant, *et al.*, 2012), and used to normalize quantitative analyses of salmonellae (Klerks, *et al.*, 2006, Von Felten, *et al.*, 2010). Detection of salmonellae by *q*PCR was based on an established protocol for end-point PCR using primers 139 ($^{5'}$GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 ($^{5'}$TCA TCG CAC CGT CAA AGG AAC C) (Rahn, *et al.*, 1992) to amplify a 284-bp-fragment of the *inv*A gene that encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by salmonellae (Suárez & Rüssmann, 1998, Khan, *et al.*, 2000). This procedure was validated and proposed as the international standard diagnostic method for quality assurance laboratories in epidemiological studies on *Salmonella* spp. (Malorny, *et al.*, 2003). SYBR Green based *q*PCR was performed in triplicate in a total volume of 20 µl containing 10 µl of Quanta Mix (Quanta BioSciences, Gaithersburg, MD), 0.2 µl of each primer 139 and 141 (100 ng μl^{-1}) and 1 µl of DNA template in an Eppendorf Mastercycler (ep realplex^2; Eppendorf, Hauppauge, NY) using an initial denaturation at 96°C for 3 minutes, and 35 cycles of denaturation at 96°C, annealing at 64°C, and extension at 72°C, each for 30 seconds. The amplification was followed by a melting curve analysis. Quantification was based on a standard curve generated from serial dilutions

of ethanol-fixed cells of *S. enterica* serovar Typhimurium ATCC 14028 quantified by epifluorescence microscopy after DAPI staining (Hahn, *et al.*, 1992).

Results and Discussion

Selection and characterization of *Salmonella* isolates All isolates were confirmed as salmonellae by the Texas Department of State Health Services (Austin, TX), with isolate S12 identified as serovar Give (PFGE pattern XB-SLGV-110 [01]), isolate S11 as serovar Thompson (PFGE pattern XB-SLTH-096 [01]), S3 as serovar Newport (PFGE pattern XB-SLGM-043 [01]) and S19 as serovar -:z10:z39 (PFGE pattern XB-SlXX-176). Serovars like Newport and Give are often found in animal feces (Wales, *et al.*, 2009, Jiménez, *et al.*, 2011), and have been linked to several outbreaks of salmonellosis in the recent past (e.g. serovars Give (Higgins, *et al.*, 1997, Girardin, *et al.*, 2006), Thompson (Linares, *et al.*, 1984, Campbell, *et al.*, 2001, Nygård, *et al.*, 2008) and Newport (Schneider, *et al.*, 2011)). Pathogenicity tests with *C. elegans* as host resulted in survival curves (Figure 3.1) that were significantly different for *C. elegans* feeding on *E. coli* OP 50 or on the *Salmonella* strains ($p<0.001$) (Table 3.1). These tests confirmed that all strains isolated from the environment remained pathogenic with mean survival time of *Salmonella*-fed *C. elegans* being lower (between 3.0 to 4.0 days) than that of *E. coli*-fed *C. elegans* (9.5 days) (Table 3.1). These values were similar to those obtained by others for different salmonellae (Aballay, *et al.*, 2000, Aballay & Ausubel, 2001) demonstrating that our isolates from aquatic biofilms were virulent despite their potentially long occurrence outside animal

hosts. This result also implies that, if they stay viable or even grow in biofilms, these environmental strains have the potential to become health hazards when detached from biofilms and dispersed into the water column.

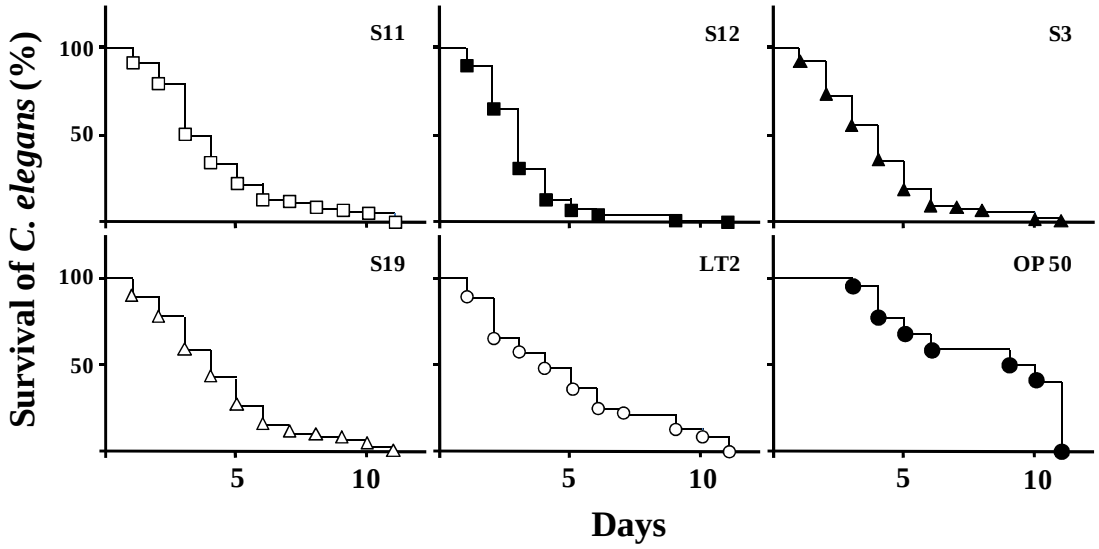


Fig. 3.1 Kaplan-Meier survival plots of *Caenorhabditis elegans* fed on *Salmonella enterica* serovar Thompson (S11, open squares) (n = 90; P< 0.0001), serovar Give (S12, closed squares) (n = 90; P< 0.0001), serovar Newport (S3, closed triangles) (n = 90; P< 0.0001), serovar -:z10:z39 (S19, open triangles) (n = 90; P< 0.0001), serovar Typhimurium (LT2, open circles) (n = 90; P< 0.0001), and *Escherichia coli* (OP 50, closed circles) (n = 90; control) for one day, with subsequent daily transfers to plates with *E. coli* OP 50.

Table 3.1 Comparison of Kaplan-Meier survival plots of *C. elegans* fed on *E. coli* OP50 and different Salmonella strains.

Strain	Mean survival (days)	P value
S. enterica serovar Thompson (isolate S11)	3.5	<0.001
S. enterica serovar Give (isolate S12)	3.0	<0.001
S. enterica serovar Newport (isolate S3)	4.0	<0.001
S. enterica serovar -:z10:z39 (isolate S19)	4.0	<0.001
S. enterica serovar Typhimurium (LT2)	4.0	<0.001
E. coli (OP 50)	9.5	-

Quantification of salmonellae in water and biofilms by *q*PCR In our mesocosm experiments, **s**almonellae could only be detected in water and biofilms when they were inoculated, but not as indigenous organisms in naturally grown biofilms during the experimental period of 4 weeks (data not shown). Salmonellae could also only be isolated from samples with inoculated strains. Isolates resembled the inoculated strains as demonstrated by identical rep-PCR patterns (data not shown). Cell numbers of strains inoculated into water at densities of about 10^6 cells ml^{-1} declined by up to 3 orders of magnitude to close to or below the detection limit of our *q*PCR protocol (i.e. 10^3 cells ml^{-1}) within 2 days (Table 3.2). Strains were not detectable (nd, Table 3.2) or inconsistently detected with low numbers in replicate samples (0, Table 3.2) afterwards. This result resembles that of others that observed rapid declines in the numbers of salmonellae after inoculation into microcosms with natural lake water (Liang, *et al.*, 1982). In sterilized lake water, however, cells persisted in high density suggesting effects of predation (Liang, *et al.*, 1982) rather than bacteriocidal effects or stress of inoculation on the decline in natural water (Klein & Alexander, 1986). This speculation is corroborated by other studies in which rapid declines of inoculated bacteria (e.g. *E. coli*, *Pseudomonas* sp., *Klebsiella pneumoniae*) in natural lake water were not meant to be caused by injury or stress (Gurijala & Alexander, 1988), but rather by predation by protozoa (Scheuerman, *et al.*, 1988).

In the initially *Salmonella*-free biofilms covered with inoculated water, numbers of salmonellae increased to maximum densities between 10^6 and 10^7 cells cm^{-2} within

a day (Table 3.2). Within two weeks, however, numbers in biofilms declined to below the detection limit similar to cells in water (Table 3.2). However, even though salmonellae were generally not detectable after 2 weeks by our molecular tools, viable cells were present. These cells grew in semi-selective media, and isolates could be obtained from water and biofilm samples 4 weeks after inoculation of the aquaria. All isolates resembled those strains inoculated as demonstrated by identical rep-PCR patterns (data not shown). These results demonstrate that inoculated strains do not establish in high numbers in water and biofilms, however, they remain detectable by growth dependent methods after semi-selective enrichment and thus were viable for the entire experimental period.

Table 3.2 *q*PCR-based detection of different *Salmonella* isolates inoculated into water containing tiles with clean biofilms in mesocosms at different time steps (mean cell numbers (± SE) $x10^3$ per cm^2 of biofilm or ml of water, respectively)

Time	Hours						Days					Weeks		
	0	12	24	36	48	60	3	4	5	6	7	2	3	4
Isolate S11 (*Salmonella enterica* serovar Thompson)														
Water	3129	767	365	85	nd	1	0	0	0	0	nd	nd	nd	nd
	(377)	(61)	(55)	(13)		(0)	(0)	(0)	(0)	(0)				
Biofilm	9	920	110	16	23	84	35	35	27	3	3	5	0	nd
	(3)	(200)	(25)	(2)	(3)	(11)	(7)	(15)	(3)	(0)	(2)	(2)	(0)	
Isolate S12 (*Salmonella enterica* serovar Give)														
Water	393	358	248	34	nd	0	nd	0	0	nd	nd	0	nd	nd
	(40)	(74)	(71)	(5)		(0)		(0)	(0)			(0)		
Biofilm	13	1163	432	28	33	78	29	73	10	0	1	nd	nd	nd
	(3)	(554)	(142)	(7)	(7)	(14)	(9)	(21)	(2)	(0)	(0)			
Isolate S3 (*Salmonella enterica* serovar Newport)														
Water	280	199	19	8	nd	nd	0	0	nd	0	nd	nd	nd	nd
	(38)	(28)	(4)	(0)			(0)	(0)		(0)				
Biofilm	0	87	1183	143	21	242	10	6	21	nd	nd	nd	nd	nd
	(0)	(48)	(524)	(67)	(5)	(109)	(4)	(2)	(17)					
Isolate S19 (*Salmonella enterica* serovar -:z10:z39)														
Water	311	51	56	68	0	nd	0	0	0	0	0	0	nd	nd
	(34)	(6)	(12)	(5)	(0)		(0)	(0)	(0)	(0)	(0)	(0)		
Biofilm	0	1650	25365	630	159	45	11	6	4	0	6	nd	0	nd
	(0)	(870)	(8953)	(186)	(68)	(12)	(2)	(1)	(1)	(0)	(2)		(0)	
***Salmonella enterica* serovar Typhimurium LT2**														

nd, not detected

A repetition of this experiment using a 10-fold higher inoculum of *S. enterica* serovar Newport showed the same pattern of population dynamics in both inoculated water and initially clean biofilm; cells, however, remained detectable for up to 4 weeks and in higher density in biofilms with 10^4-10^5 cells cm^{-2} (Table 3.3). The same pattern of population dynamics was observed when cells were inoculated into biofilms which were then covered with clean water. Cell densities of about 2 x 10^6 cells cm^{-2} in biofilms declined after 2 days by about one order of magnitude, but remained detectable for 4 weeks with densities between 10^4 to 10^5 cells cm^{-2} (Table

3.3). Cells in water were only detected during the initial 2 days, with numbers that remained close to the detection limit. Because numbers of *Salmonella* in water remained far below the presumed infective dose of 10^5 cells (Kothary & Babu, 2001), it seems unlikely that biofilms in natural waters act as reservoirs for subsequent water contamination events. This might be different in distribution systems for drinking or irrigation water where biofilms are often found in distribution pipes (September, *et al.*, 2007, Pachepsky, *et al.*, 2012). Then, detachment or significant disturbance of these biofilms might result in a pulse release of pathogens with numbers that then rise above the infective dose if present.

Table 3.3 *q*PCR-based quantification of *Salmonella enterica* serovar Newport inoculated into water or biofilm samples in mesocosms at different times steps (cell numbers x10^3 per cm^2 of biofilm or ml of water, respectively)

Time	**Hours**					**Days**					**Weeks**		
	0	**12**	**24**	**36**	**48**	**3**	**4**	**5**	**6**	**7**	**2**	**3**	**4**
Treatment 1: inoculated water													
Water	11513	5539	11925	3030	38	5	2	0	9	3	2	nd	nd
	(2768)	(2037)	(3713)	(1497)	(16)	(1)	(0)	(0)	(5)	(2)	(1)		
Treatment 2: inoculated water, clean biofilm													
Water	10167	18746	4089	61	33	2	nd	nd	nd	nd	2	1	nd
	(1671)	(3643)	(395)	(32)	(13)	(2)					(1)	(0)	
Biofilm	14	3373	10383	11767	22301	453	762	1320	303	836	183	40	27
	(4)	(757)	(1402)	(1867)	(4183)	(77)	(196)	(323)	(27)	(75)	(27)	(9)	(8)
Treatment 3: clean water, inoculated biofilm													
Water	1	16	13	9	nd	nd	nd	nd	0	nd	0	nd	nd
	(0)	(3)	(2)	(3)					(0)		(0)		
Biofilm	2216	2020	2175	3432	7304	521	53	208	144	347	164	21	19
	(514)	(445)	(387)	(427)	(1180)	(53)	(5)	(44)	(27)	(58)	(12)	(4)	(4)

nd, not detected

Although *Salmonella* can survive in aquatic biofilms as demonstrated in this and other studies (Arnon, *et al.*, 1997), it is still unclear whether they can actually grow or just persist. While biofilms are regarded as hot spots of rapidly available carbon resources (Geesey, *et al.*, 1978, Augspurger, *et al.*, 2008), that could allow growth of heterotrophic organisms such as *Salmonella*, the pattern of population dynamics of *Salmonella* with increasing or constant cell numbers for a few days, followed by rapid declines and a final long tailing phase with low and variable cell numbers, does not support any speculations on growth. Since more nutrient rich environments such as dairy lagoons and field soil support the same pattern of population dynamics of *Salmonella* (Toth, *et al.*, 2011), and survival of *Salmonella* in estuarine water was not affected by dissolved organic and inorganic components (Chandran & Hatha, 2005), we think that biofilms more likely increase the survival of salmonellae by reducing environmental stress such as predation pressure (Johnson, 2008); however, the results also allow for speculations on a combination of both growth and predation. Future studies should therefore address questions on potential growth or persistence of *Salmonella* in environmental biofilms in more detail, and investigate the impact of potential pulse releases of these pathogens from biofilms in irrigation systems.

Acknowledgements

The authors are grateful for financial support from Texas State University-San Marcos, the Department of Biology, and the State of Texas through the American Recovery and Reinvestment Act (ARRA).

References

[1] Aballay A & Ausubel FM (2001) Programmed cell death mediated by ced-3 and ced-4 protects *Caenorhabditis elegans* from *Salmonella typhimurium*-mediated killing. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 2735-2739.

[2] Aballay A, Yorgey P & Ausubel FM (2000) *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Current Biology* **10**: 1539-1542.

[3] Arnon R, Starosvetzky J, Arbel T & Green M (1997) Survival of *Legionella pneumophila* and *Salmonella typhimurium* in biofilm systems. *Water Science and Technology* **35**: 293-300.

[4] Augspurger C, Gleixner G, Kramer C & Küsel K (2008) Tracking carbon flow in a 2-week-old and 6-week-old stream biofilm food web. *Limnology and Oceanography* **53**: 642-650.

[5] Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71-94.

[6] Byappanahalli MN, Shively DA, Nevers MB, Sadowsky MJ & Whitman RL (2003) Growth and survival of *Escherichia coli* and enterococci populations in the macro-alga *Cladophora* (Chlorophyta). *FEMS Microbiology Ecology* **46**: 203-211.

[7] Byappanahalli MN, Sawdey R, Ishii S, Shively DA, Ferguson JA, Whitman RL & Sadowsky MJ (2009) Seasonal stability of *Cladophora*-associated *Salmonella* in Lake Michigan watersheds. *Water Research* **43**: 806-808.

[8] Campbell JV, Mohle-Boetani J, Reporter R, *et al.* (2001) An outbreak of *Salmonella* serotype Thompson associated with fresh cilantro. *Journal of Infectious Diseases* **183**: 984-987.

[9] Chandran A & Hatha AAM (2005) Relative survival of *Escherichia coli* and *Salmonella typhimurium* in a tropical estuary. *Water Res*earch **39**: 1397-1403.

[10] Cherry WB, Thomason BM, Gladden JB, Holsing N & Murlin AM (1975) Detection of *Salmonella* in foodstuffs, feces, and water by immunofluorescence. *Annals of the New York Academy of Sciences* **254**: 350-368.

[11] Cherry WB, J. B. Hanks, B. M. Thomason, A. M. Murlin, J. W. Biddle a & Croom JM (1972) Salmonellae as an index of pollution of surface waters. *Applied Microbiology* **24**: 334–340.

[12] Cote C & Quessy S (2005) Persistence of *Escherichia coli* and *Salmonella* in surface soil following application of liquid hog manure for production of pickling cucumbers. *Journal of Food Protection* **68**: 900-905.

[13] Danyluk MD, Nozawa-Inoue M, Hristova KR, Scow KM, Lampinen B & Harris LJ (2008) Survival and growth of *Salmonella Enteritidis* PT 30 in almond orchard soils. *Journal of Applied Microbiology* **104**: 1391-1399.

[14] Domingo JWS, Fuentes FA & Hazen TC (1989) Survival and activity of *Streptococcus faecalis* and *Escherichia coli* in petroleum contaminated tropical marine waters. *Environmental Pollution* **56**: 263-281.

[15] Gaertner JP, Forstner MRJ, Rose FL & Hahn D (2008) Detection of salmonellae in different turtle species within a headwater spring ecosystem. *Journal of Wildlife Diseases* **44**: 519-526.

[16] Gaertner JP, Mendoza JA, Forstner MRJ & Hahn D (2011) Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. *Journal of Water and Health* **9**: 458-466.

[17] Gaertner JP, Garres T, Becker JC, Jimenez ML, Forstner MRJ & Hahn D (2009) Temporal analyses of salmonellae in a headwater spring ecosystem reveals the effect of precipitation and runoff events. *Journal of Water and Health* **7**: 115-121.

[18] Geesey GG, Mutch R, Costerton JW & Green RB (1978) Sessile bacteria-important component of microbial population in small mountain streams. *Limnology and Oceanography* **23**: 1214-1223.

[19] Girardin F, Mezger N, Hächler H & Bovier PA (2006) *Salmonella* serovar Give: an unusual pathogen causing splenic abscess *European Journal of Clinical Microbiology & Infectious Diseases* **25**: 272-274.

[20] Gurijala KR & Alexander M (1988) Role of sublethal injury in decline of bacterial populations in lake water. *Applied and Environmental Microbiology* **54**: 2859-2861.

[21] Hahn D, Amann RI, Ludwig W, Akkermans ADL & Schleifer K-H (1992) Detection of microorganisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. *Journal of General Microbiology* **138**: 879-887.

[22] Higgins R, Désilets A, Cantin M, *et al.* (1997) Outbreak of *Salmonella* Give in the province of Quebec. *The Canadian Veterinary Journal* **38**: 780-781.

[23] Hoelzer K, Moreno Switt AI & Wiedmann M (2011) Animal contact as a source of human non-typhoidal salmonellosis. *Veterinary Research* **42**: 34-62.

[24] Humphrey T (2000) *Public-health aspects of Salmonella infection*. CABI Publishing, Wallingford, UK.

[25] Ishii S, W.B. K, Hicks RE & Sadowsky MJ (2006) Presence and growth of naturalized *Escherichia coli* in temperate soils from Lake Superior watersheds. *Applied and Environmental Microbiology* **72**: 612-621.

[26] Ishii S, Yan T, Shively DA, Byappanahalli MN, Whitman RL & Sadowsky MJ (2006) *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. *Applied and Environmental Microbiology* **72**: 4545-4553.

[27] Jiménez L, Muñiz I, Toranzos GA & Hazen TC (1989) Survival and activity of *Salmonella typhimurium* and *Escherichia coli* in tropical freshwater. *Journal of Applied Microbiology* **67**: 61-69.

[28] Jiménez M, Martínez-Urtaza J & Chaidez C (2011) Geographical and temporal dissemination of salmonellae isolated from domestic animal hosts in the Culiacan Valley, Mexico. *Microbial Ecology* **61**: 811-820.

[29] Johnson LR (2008) Microcolony and biofilm formation as a survival strategy for bacteria. *Journal of Theoretical Biology* **251**: 24-34.

[30] Khan AA, Navaz MS, Khan SA & Cerniglia CE (2000) Detection of multidruge-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. *FEMS Microbiology Letters* **182**: 355-360.

[31] Klein TM & Alexander M (1986) Bacterial inhibitors in lake water. *Applied and Environmental Microbiology* **52**: 114-118.

[32] Klerks MM, van Bruggen AH, Zijlstra C & Donnikov M (2006) Comparison of methods of extracting Salmonella enterica serovar Enteritidis DNA from environmental substrates and quantification of organisms by using a general internal procedural control. *Applied and Environmental Microbiology* **72**: 3879-3886.

[33] Kothary MH & Babu US (2001) Infective dose of foodborne pathogens in volunteers: a review. *Journal of Food Safety* **21**: 49-73.

[34] Ksoll WB, Ishii S, Sadowsky MJ & Hicks RE (2007) Presence and sources of fecal coliform bacteria in epilithic periphyton communities of Lake Superior. *Applied and Environmental Microbiology* **73**: 3771-3778.

[35] Labrousse A, Chauvet S, Couillault C, Kurz CL & Ewbank JJ (2000) *Caenorhabditis elegans* is a model host for *Salmonella typhimurium*. *Current Biology* **10**: 1543-1545.

[36] Liang LN, Sinclair JL, Mallory LM & Alexander M (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. *Applied and Environmental Microbiology* **44**: 708-714.

[37] Linares AP, Cohen SH, Goldstein E, Kelley ADK & Eisenstein TK (1984) Febrile gastroenteritis due to *Salmonella* Thompson—Report of an Outbreak. *The Western Journal of Medicine* **141**: 203-205.

[38] Malorny B, Hoorfar J, Bunge C & Helmuth R (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards and international standard. *Applied and Environmental Microbiology* **69**: 290-296.

[39] Marsollier L, Stinear T, Aubry J, *et al.* (2004) Aquatic plants stimulate the growth of and biofilm formation by *Mycobacterium ulcerans* in axenic culture and harbor these bacteria in the environment. *Applied and Environmental Microbiology* **70**: 1097-1103.

[40] Martinez-Urtaza J, Saco M, de Novoa J, Perez-Pineiro P, Peiteado J, Lozano-Leon A & Garcia-Martin O (2004) Influence of environmental factors and human activity on the presence of *Salmonella* serovars in a marine environment. *Applied and Environmental Microbiology* **70**: 2089-2097.

[41] Nygård K, Lassen J, Vold L, *et al.* (2008) Outbreak of *Salmonella* Thompson infections linked to imported rucola lettuce. *Foodborne Pathogens and Disease* **5**: 165-173.

[42] Pachepsky Y, Morrow J, Guber A, Shelton D, Rowland R & Davies G (2012) Effect of biofilm in irrigation pipes on microbial quality of irrigation water. *Letters in Applied Microbiology* **54**: 217-224.

[43] Purevdorj B (2002) Hydrodynamic considerations of biofilm structure and behavior. *Microbial Biofilms* (Ghannoum MA & O'Toole G, eds.). pp. 160-173. ASM Press.

[44] Rahn K, De Grandis SA, Clarke RC, *et al.* (1992) Amplification of an invA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes* **6**: 271-279.

[45] Samant S, Sha Q, Iyer A, Dhabekar P & Hahn D (2012) Quantification of *Frankia* in soils using SYBR Green based *q*PCR. *Systematic and Applied Microbiology* **35**: 191-197.

[46] Sanyal D, Douglas T & Roberts R (1997) *Salmonella* infection acquired from reptilian pets. *Archives of Diseases in Childhood* **77**: 345-346.

[47] Scheuerman PR, Schmidt JP & Alexander M (1988) Factors affecting the survival and growth of bacteria introduced into lake water. *Archives of Microbiology* **150**: 320-325.

[48] Schneider JL, White PL, Weiss J, *et al.* (2011) Multistate outbreak of multidrug-resistant *Salmonella* Newport infections associated with ground beef, October to December 2007. *Journal of Food Protection* **74**: 1315-1319.

[49] Semenov AV, van Overbeek L & van Bruggen AH (2009) Percolation and survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in soil amended with contaminated dairy manure or slurry. *Applied and Environmental Microbiology* **75**: 3206-3215.

[50] September SM, Els FA, Venter SN & Broezel VS (2007) Prevalence of bacterial pathogens in biofilms of drinking water ditribution systems. *Journal of Water and Helath* **5**: 219-227.

[51] Sha Q, Gunathilake A, Forstner MRJ & Hahn D (2011) Temporal analyses of the distribution and diversity of *Salmonella* in natural biofilms. *Systematic and Applied Microbiology* **34**: 353-359.

[52] Suárez M & Rüssmann H (1998) Molecular mechanisms of *Salmonella* invasion: the type III secretion system of the pathogenicity island 1. *Interntional Microbiology* **1**: 197-204.

[53] Tauxe RV (1997) Emerging foodborne diseases: An evolving public health challenge. *Emerging Infectious Diseases* **3**: 425-434.

[54] Topp E, Welsh M, Tien YC, Dang A, Lazarovits G, Conn K & Zhu H (2003) Strain-dependent variability in growth and survival of *Escherichia coli* in agricultural soil. *FEMS Microbiology Ecology* **44**: 303-308.

[55] Toth JD, Aceto HW, Rankin SC & Dou Z (2011) Survival characteristics of *Salmonella enterica* serovar Newport in the dairy farm environment. *Journal of Dairy Science* **94**: 5238-5246.

[56] Von Felten A, Defago G & Maurhofer M (2010) Quantification of *Pseudomonas* fluorescens strains F113, CHA0 and Pf153 in the rhizosphere of maize by strain-specific real-time PCR unaffected by the variability of DNA extraction efficiency. *Journal of Microbiological Methods* **81**: 108-115.

[57] Wales AD, McLaren IM, Bedford S, Carrique-Mas JJ, Cook AJ & Davies RH (2009) Longitudinal survey of the occurrence of *Salmonella* in pigs and the environment of nucleus breeder and multiplier pig herds in England. *The Veterinary Records* **165**: 648-657.

[58] Watnick PI & Kolter R (1999) Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Molecular Microbiology* **34**: 586-595.

[59] Wells EV, Boulton M, Hall W & Bidol SA (2004) Reptile-associated salmonellosis in preschool-aged children in Michigan, January 2001-June 2003. *Clinical Infectious Diseases* **39**: 687-691.

[60] Woodward DL, Khakhira R & Johnson WM (1997) Human salmonellosis associated with exotic pets. *Journal of Clinical Microbiology* **35**: 2786-2790.

[61] World Health Organization (2002) *The world health report. Reducing risks, promoting healthy life*. World Health Organization, Geneva, Switzerland.

[62] Yildiz FH & Schoolnik GK (1999) Vibrio cholerae O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 4028-4033.

[63] You Y, Rankin SC, Aceto HW, Benson CE, Toth JD & Dou Z (2006) Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Applied and Environmental Microbiology* **72**: 5777-5783.

[64] Zachow C, Pirker H, Westendorf C, Tilcher R & Berg G (2009) The *Caenorhabditis elegans* assay: a tool to evaluate the pathogenic potential of bacterial biocontrol agents. *European Journal of Plant Pathology* **125**: 367-376.

CHAPTER 4

DIVERSITY AND ABUNDANCE OF *SALMONELLA* IN BIOFILMS AND WATER IN A HEADWATER ECOSYSTEM

Sha, Q., M.R.J. Forstner, D. Hahn. Diversity and abundance of Salmonella in biofilms and water in a headwater ecosystem. FEMS Microbiology Ecology (**in press**).

Abstract

The diversity and abundance of *Salmonella* was analyzed in biofilm and water samples from the spring and slough arms of Spring Lake, the headwaters of the San Marcos River, Texas, during the drought of 2011, with only one potential run-off event at the beginning of the study. Salmonellae were detected in semi-selective enrichment cultures by end-point PCR during the entire sampling period (11 sampling events during 2 months), with higher frequency at the spring arm site compared to the slough arm site. From the spring arm site, 73% of the biofilms and 41% of the water samples were positive for salmonellae, while only 9% of the biofilms and 23% of the water samples were positive from the slough arm site. Salmonellae could be isolated from all positive samples, with higher diversity in biofilms compared to water samples, and more strains obtained from the spring arm (21 and 6 strains in biofilms and water, respectively) than from the slough arm (8 and 5 strains). A significant positive correlation was discovered between numbers of isolates and diversity. Differences between sites were generally caused by less frequently detected isolates, while the majority of isolates that were present in both biofilms and water from both sites was represented by three strains only (serovars Montevideo, Newport and Gaminara, respectively). Quantification attempts by *q*PCR directly in samples without prior enrichment did not result in a reliable detection of salmonellae, suggesting that numbers in all samples were below the detection limit (10^3 cells per 500 ml of water or 2.56 cm^{-2} of biofilm). These results indicate long-term persistence of *Salmonella* at

considerable diversity, albeit in low numbers, in both water and heterogeneous aquatic biofilms, in the absence of concurrent runoff that could be expected to contribute to contamination.

Introduction

Members of the genus *Salmonella* represent important enteric pathogens that are typically transmitted to humans via food and drinking water contaminated with feces of vertebrate animals (Islam, *et al.*, 2004, Krtinic, *et al.*, 2010, Levantesi, *et al.*, 2012). Animals are well-known reservoirs for salmonellae (Johnson-Delaney, 1996, Refsum, *et al.*, 2002, Doyle & Erickson, 2006), and many studies have demonstrated their significance in salmonellosis in humans (Anonymous, 1995, Anonymous, 1999, Mermin, *et al.*, 2004, Dallap Schaer, *et al.*, 2010). The intestinal tract of vertebrates is typically assumed to be the native habitat of salmonellae (Woodward, *et al.*, 1997), with feces released then contaminating the environment (Natvig, *et al.*, 2002, Holley, *et al.*, 2008). It is known that salmonellae released by animals in the vicinity of aquatic systems represent a potent non-point source of contamination for water and sediments when transported into the aquatic system by strong rainfall events and associated runoff (Kinzelman, *et al.*, 2004, Arnone & Perdek Walling, 2007). Non-point sources include agricultural run-off, contaminated soils surrounding the system, and fecal droppings from wildlife (Kinzelman, *et al.*, 2004) and domesticated animals (Veling, *et al.*, 2002). Released from animal reservoirs into the environment, salmonellae have been shown to survive, e.g., in cattle manure (Kearny, *et al.*, 1993,

Himathongkham, *et al.*, 1999) or in soils (Islam, *et al.*, 2004, Cote & Quessy, 2005, Franz, *et al.*, 2005) for time periods that exceeded a month.

Recent studies in our laboratory frequently detected *Salmonella* sp. in water, sediments, animals (i.e., fish, turtles) and biofilms even in supposedly clean habitats such as Spring Lake, the spring-fed headwaters of the San Marcos River, Texas (Gaertner, *et al.*, 2008, Gaertner, *et al.*, 2008, Gaertner, *et al.*, 2008, Gaertner, *et al.*, 2009, Gaertner, *et al.*, 2011, Sha, *et al.*, 2011). Salmonellae were detected in natural biofilms on concrete surfaces with a significant micro-heterogeneity and differences in diversity of viable strains (Sha, *et al.*, 2011). Isolates detected repeatedly over time in natural biofilms at different sites remained pathogenic and were shown to persist and stay viable in mesocosm studies in biofilms and water in high numbers for some time (Sha, *et al.*, 2011). These data suggested that the current paradigm that defines salmonellae as a contaminant might need to be revised to an updated version that includes salmonellae as an ecosystem component.

The goal of this study was to assess the potential colonization of aquatic biofilms by salmonellae over time. For this purpose, natural biofilms grown on ceramic tiles with defined surface area and devoid of salmonellae were introduced into water at two sites in Spring Lake. Tiles were removed frequently during a 2-month period for qualitative and quantitative analyses of salmonellae in adhering biofilms. Analysis methods included end-point PCR after semi-selective enrichments of salmonellae for their detection, isolation from semi-selective enrichments and characterization of

isolates by rep-PCR for diversity assessments of salmonellae, and finally quantitative PCR (*q*PCR) for the determination of the abundance of salmonellae directly in the environmental samples.

Material and Methods

Experimental setup and sample preparation

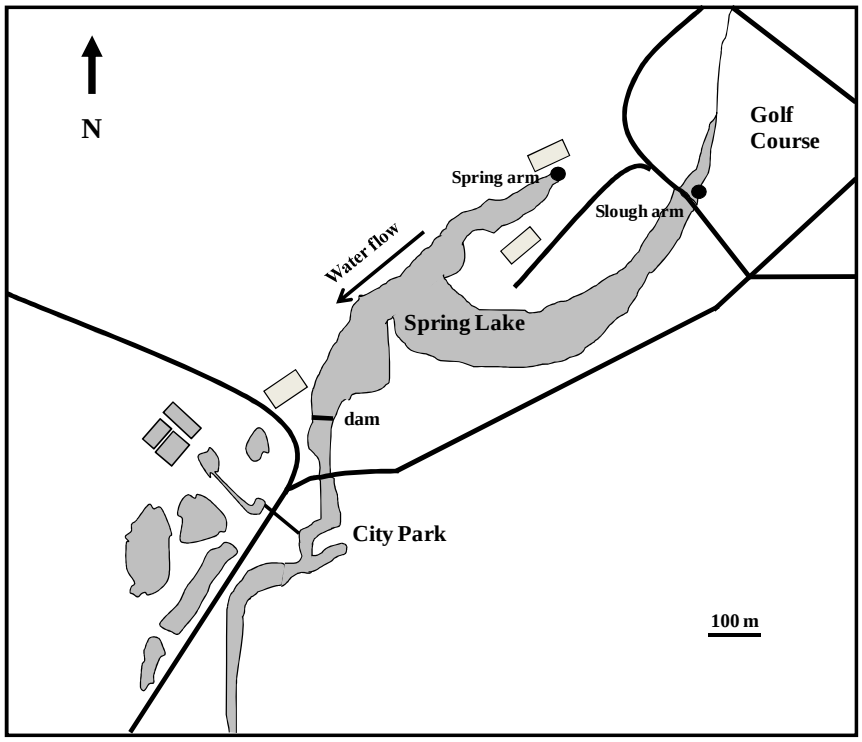


Figure 4.1 Schematic presentation of sampling sites (black dots) in Spring Lake (San Marcos, TX), i.e. its spring arm (29.894128, 97.929839), and its slough arm (29.893736, 97.927456). Dark lines represent roads and rectangles buildings.

Biofilms were grown on clean ceramic tiles (2.2 x 2.2 cm, non-glazed) in a stream channel with running spring water for 8 months. Biofilms on 10 haphazardly selected tiles were checked for salmonellae by PCR after semi-selective enrichment (see below), and all remaining biofilms assumed to be free of salmonellae when these 10 biofilms remained negative. On May 30, 2011, each 250 tiles with biofilms were

transferred into water at 2 sites in Spring Lake (San Marcos, TX), i.e. its spring arm (29.894128, 97.929839), and its slough arm (29.893736, 97.927456) (Figure 4.1). Spring Lake is generally considered one of the most pristine waters in Texas fed by a system of 200 artesian springs of the Edwards Aquifer (Slattery & Fahlquist, 1997). The sampling site "spring arm" was located just upstream of these springs and surrounded by concrete walkways and buildings, while the second site "slough arm" was downstream of the Sink Creek discharge area and surrounded by a golf course. Both sites represented lentic environments with virtually no flow.

Tiles were left on concrete stairs at the spring arm site and on shore sediments in the slough arm, at a water depth of about 10 cm. Sampling started about 1 month after deposition of the tiles, which coincided with the only precipitation event (46 mm between the first and second sampling June 21 and 22, respectively) during the 2-month sampling period (June 21 to August 25, 2011) with a total of 11 sampling events. Precipitation before deposition of the tiles on May 30 consisted of 27 mm, 9 mm and 14 mm water on May 12, May 20 and May 21, respectively, and no significant precipitation the previous three months. At each sampling event, basic environmental characteristics (pH, temperature, conductivity and dissolved oxygen) were determined in water using a Hydrolab^{TM} DS5 multiprobe sonde (Hach Environmental, Loveland, CO). The pH, temperature and conductivity were similar between sites and stable during the entire study with values for the pH of 7.2 ± 0.2 for both sites, temperatures of 22.5 ± 0.6°C and 24.0 ± 0.5°C, and conductivities of 505 ±

19 µS cm^{-2} and 497 ± 39 µS cm^{-2} for the spring and slough arms, respectively. Dissolved oxygen concentrations, however, were different between sites, but again relatively stable during the entire study with higher concentrations at the spring arm site (3.6 ± 1.3 ppm) compared to the slough arm site (1.6 ± 1.2 ppm).

At each sampling event, 10 tiles were collected from each site, transferred individually to 50 ml Falcon tubes and covered with 20 ml PBS buffer (0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.2). Two 500 ml water samples were also collected from each site. Water samples were filtered through 0.2 µm Whatman Nuclepore Track-Etched membranes, and the filter placed into 50 ml Falcon tubes containing 20 ml of PBS buffer. Cells were released from tiles or filters by sonication in a Fisher sonic cleaner (2QT; Fisher Scientific Inc., PA) for 10 minutes. Tiles or filters were removed afterwards, and released cells collected by centrifugation at 4,400 x g for 15 minutes. Cell pellets were resuspended in 1 ml of sterile distilled water, and each 3 subsamples of 100 µl then used for semi-selective enrichment and isolation of salmonellae, and for quantification by *q*PCR.

Detection of salmonellae

For semi-selective enrichment and isolation of salmonellae, 100 µl subsamples were added to 2-ml cryo-tubes containing 1 ml of Buffered Peptone Water (BPW; L^{-1}: 10 g peptone, 5 g NaCl, 9 g Na_2HPO_4, 1.5 g KH_2PO_4, pH 7.2) (International Standard Organization, 1993) and incubated at 37ºC for 24 hours. After incubation, 100 µl of these cultures were transferred to cryo-tubes containing 1 ml of

Rappaport–Vassiliadis (RVS) broth (L^{-1}: 4.5 g soybean peptone, 29 g $MgCl_2 \cdot 7\ H_2O$, 8 g NaCl, 0.4 g K_2HPO_4, 0.6 g KH_2PO_4, 0.036 g malachite-green, pH 5.2) for semi-selective enrichment of salmonellae (Vassiliadis, *et al.*, 1981) and incubated at 37ºC for 48 hours. Sub-samples (100 µl) were then transferred to cryo-tubes with fresh RVS medium for a second enrichment at 37ºC for 48 hours. These enrichments were subsequently screened for the presence of salmonellae by end-point PCR detecting a 284-bp-fragment of the *inv*A gene as described previously (Hahn, *et al.*, 2007). PCR products were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 µg ml^{-1}) (Sambrook, *et al.*, 1989).

Diversity assessment of salmonellae

Sub-samples (100 µl) of the second enrichment were also plated on RVS agar (RVS solidified with 15 g agar L^{-1}) and incubated for 16 hours. From each sample, 10 colonies were chosen haphazardly, incubated in Luria–Bertani broth (LB; L^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) at 37ºC for 7 hours, and identified as salmonellae by the detection of the *inv*A gene fragment by end-point PCR (Hahn, *et al.*, 2007). Isolates representing salmonellae were further characterized by rep-PCR as described in (Hahn, *et al.*, 2007). Banding profiles were screened visually by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook, *et al.*, 1989), and representative profiles documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA) (Sha, *et al.*, 2011).

Quantification of salmonellae

For the quantification of salmonellae by *q*PCR, cell pellets from biofilm and water samples were lysed in 100 µl of 50 mM NaOH at 65℃for 30 minutes. Cell lysates of biofilms were cleaned using the SurePrep Soil DNA Isolation kit (Fisher Scientific, Pittsburgh, PA) after the addition of defined amounts (1 µl) of DNA of the nitrogen-fixing symbiont *Frankia* Ag45/Mut15. *q*PCR quantification of *Franki*a DNA using primer set *nif*Hf1(5' GGC AAG TCC ACC ACC CAG C)/*nif*Hr158 ($^{5'}$GAC GCA CTT GAT GCC CCA) targeting the *nif*H gene of frankiae (Samant, *et al.*, 2012) before and after extraction was used to estimate extraction efficiencies for each sample. Extraction efficiencies were used to correct abundance estimates for salmonellae.

Detection and quantification of salmonellae in samples was achieved by SYBR Green based *q*PCR performed in triplicate in a total volume of 20 µl containing 10 µl of Quanta Mix (Quanta BioSciences, Gaithersburg, MD), 0.2 µl of each primer 139 and 141 (100 ng μl^{-1}) (Rahn, *et al.*, 1992) and 1 µl of DNA template in an Eppendorf Mastercycler (ep realplex^2; Eppendorf, Hauppauge, NY) using an initial denaturation at 96°C for 3 minutes, and 35 cycles of denaturation at 96°C, annealing at 64°C, and extension at 72°C, each for 30 seconds (Sha, *et al.*, 2013). The amplification was followed by a melting curve analysis. Quantification was based on a standard curve generated from serial dilutions of ethanol-fixed cells of *Salmonella enterica* serovar Typhimurium strain ATCC 14028 quantified by epifluorescence microscopy (Eclipse

80i microscope; Nikon, Lewisville, TX) after staining with 4',6-Diamidino-2-phenylindole (DAPI) (Hahn, *et al.*, 1992).

Statistics

The Pearson's correlation coefficient was calculated for the analysis of the relationship between numbers of isolates and their diversity. Analysis of Variance (ANOVA) was used to compare the difference in numbers of isolates among different sources. A $P<0.05$ was used to determine significant differences.

Results and Discussion

Detection of salmonellae

Salmonellae were detected by end-point PCR in semi-selective enrichment cultures from both biofilm and water samples throughout the study period, with generally more samples being positive for salmonellae from the "spring arm" site than from the "slough arm" site (Table 4.1- 3). Biofilm samples from the "spring arm" site were positive for salmonellae at all sampling times, with generally high percentages of detection at each sampling event (i.e. 7 to 10 biofilms from 10 tiles positive for salmonellae) (Table 4.1). Detection of salmonellae in the corresponding water samples was less frequent, with no detection of salmonellae at 3 sampling events, and generally only 1 out of 2 samples positive for the remaining 8 sampling events (Table 4.2). Biofilm and water samples from the "slough arm" site had lower salmonellae detections, with salmonellae being detected at less than 50% of the sampling times and in the samples per sampling event (Table 4.3). These results are similar to those

of previous studies that demonstrated a higher prevalence of detection in spring arm samples compared to slough arm samples (Gaertner, *et al.*, 2011).

In contrast to this and other studies (Gaertner, *et al.*, 2009, Haley, *et al.*, 2009, Gaertner, *et al.*, 2011), however, prevalence of detection in water could not be related to precipitation events. Salmonellae were detected in water samples with up to four different strains per sampling date even without precipitation for more than a month (Table 4.2 and 4.3). This result might be related to the much larger water volumes used for analysis in this study (500 ml compared to 40 ml in previous studies) allowing the enrichment of even very small numbers of salmonellae in water. Cells of salmonellae were shown to remain viable and detectable in water for several weeks by semi-selective enrichment even though salmonellae introduced into water declined very quickly (Sha, *et al.*, 2013). Thus, while presumably introduced in high numbers by runoff and therefore detectable in small samples shortly after precipitation only, our larger sampling volume enabled us to demonstrate the presence of viable strains in water even for an extended period of time after a runoff event and absent new precipitation.

Table 4.1 Diversity and abundance of *Salmonella* isolates in biofilm samples from the spring arm of Spring Lake

Sampling date	Biofilms positive for salmonellae (%, n=10)	Number of isolates	Isolates from biofilms (rep-PCR profile)																							
			1	2	3	4	5	6	7	8	9	10	12	13	14	15	22	23	24	25	26	27	28	29	30
June 21	100	75	4	3			64			1	1			1	1										
22	80	64					54		1				9												
24	100	91					77								10		4								
27	90	70					16		7						33			3	9	1	1				
July 04	90	88		50			7								31										
11	70	68		43			18								7										
13	40	39		24	5		10																		
18	10	10					5								5										
25	90	75		6	4	4	1	1							56		1					2			
August 01	100	88		16			12					10			49								1		
25	30	26													25										1
		694	4	142	9	4	264	1	8	1	1	10	9	1	217	0	5	3	9	1	1	2	1	0	1

Salmonellae in the water column can be source for contamination of heterogeneous aquatic biofilms. However, since our analyses required destructive sampling, with the consequence that different samples were analyzed in time, we can only speculate about the time and mode of contamination. If the relatively small precipitation event (9 and 14 mm water on May 20 and May 21) prior to deposition of the tiles is considered a potential runoff event and salmonellae in the water column can act as a source for colonization for at least 10 days after the runoff events, all biofilms with subsequent *Salmonella* detections might have been colonized immediately after their deposition into the water on May 30. Subsequent detection would indicate that these salmonellae could at least persist in biofilms through the end of the study 3 months later. An alternative to this scenario would be a recurring colonization of biofilms over time through deposition of salmonellae from the water

column without runoff events. This latter scenario requires either the persistence of salmonellae in the water column, a recurring contamination by salmonellae released from biofilms, or another unknown continuous source for contamination.

Table 4.2 Diversity and abundance of *Salmonella* isolates in water samples from the spring arm of Spring Lake

Sampling date	Water positive for Salmonellae (%, n=2)	Number of isolates	Isolates from water (rep-PCR profile)																							
			1	2	3	4	5	6	7	8	9	10	12	13	14	15	22	23	24	25	26	27	28	29	30
June 21	100	15		2			11					2													
22	50	8													4	4									
24	0	0																							
27	0	0																							
July 04	50	10													10										
11	50	5		4											1										
13	50	2		2																					
18	50	10					10																		
25	50	10													10										
August 01	50	10		4					6																
25	0	0																							
		70	0	12	0	0	21	0	7	0	0	2	0	0	25	4	0	0	0	0	0	0	0	0	0

Diversity assessment of salmonellae

A total of 887 isolates confirmed as salmonellae by the detection of *inv*A gene fragments by end-point PCR were obtained from both sites, with higher numbers from the “spring arm” site (764 isolates) than from the “slough arm” site (123 isolates), and from biofilms (694 and 82 isolates for the spring and slough arm sites, respectively) than from water (70 and 41 isolates, respectively) (Table 4.1- 3). Overall, 30 rep-PCR patterns were identified, with higher diversity in “spring arm” samples (23 patterns) compared to “slough arm” samples (9 patterns), and in biofilm samples (21 and 8

patterns, respectively) compared to water samples (6 and 5 patterns, respectively). These results are consistent with a previous study at the same sites in which 1 *Salmonella* strain was retrieved from water compared to 11 strains from biofilms, and 1 strain from the slough arm compared to 9 strains from the spring arm of Spring Lake (Gaertner, *et al.*, 2011).

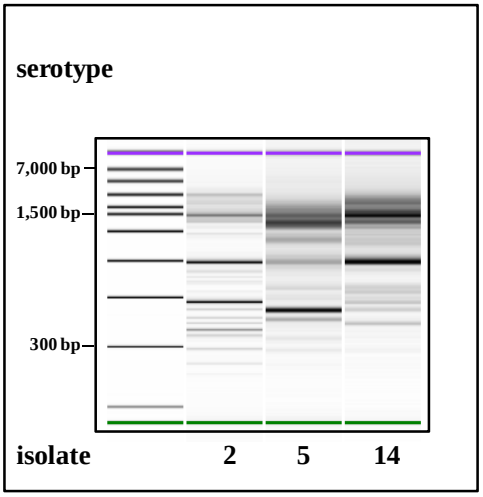


Figure 4.2 rep-PCR of the major *Salmonella* isolates present in both biofilms and water at both sites in Spring Lake (San Marcos, TX). Three strain profiles are provided (serotypes Montevideo, Newport and Gaminara, respectively), as documented using an Agilent 2100 Bioanalyzer and the DNA 7500 Kit (Agilent Technologies, Foster City, CA). Fragment sizes on the left represent those determined by the Bioanalyzer.

Table 4.3 Diversity and abundance of *Salmonella* isolates in biofilm or water samples from the slough arm of Spring Lake

Sampling date	Biofilms positive for salmonellae (%, n=10)	Number of isolates	Isolates from biofilms (rep-PCR)								
			2	5	11	14	16	18	19	20	21
June 21	10	9		7	2						
22	30	16					2	5	8	1	
24	30	29				19			10		
27	20	20				10			10		
July 04	0	0									
11	0	0									
13	0	0									
18	0	0									
25	0	0									
August 01	10	8	6			2					
25	0	0									
		82	6	7	2	31	2	5	28	1	0

Sampling date	Water positive for salmonellae (%, n=2)	Number of isolates	Isolates from biofilms (rep-PCR)								
			2	5	11	14	16	18	19	20	21
June 21	50	5	4			1					
22	0	0									
24	100	19		1		10		2			6
27	0	0									
July 04	0	0									
11	0	0									
13	50	9				9					
18	0	0									
25	0	0									
August 01	50	8	8								
25	0	0									
		41	12	1	0	21	0	2	0	0	6

A significant positive correlation was discovered between numbers of isolates and diversity ($r=0.8$, $P<0.001$). Most rep-PCR patterns were obtained at low abundance (i.e. in 1 to 10 isolates), and often only at 1 or 2 sampling times. The exception were three strains that were detected at both sites and at the majority of salmonellae

positive sampling events in both biofilm and water samples (isolates 2, 5 and 14) (Table 4.1- 3). Characterization by serotyping and pulsed-field gel electrophoresis (PFGE) at the Texas Department of State Health Services (Austin, TX) identified isolate 2 as *Salmonella enterica* serovar Montevideo (PFGE pattern XB-SLMV-304 (01)), isolate 5 as *S. enterica* serovar Newport (XB-SLNP-731), and isolate 14 as *S. enterica* serovar Gaminara (XB-SLGM-022) (Figure 4.2). While our enrichment method could introduce a bias towards the detection of the most abundant strains, the analysis of 10 replicate colonies per biofilm sample and of 10 biofilm samples per site and sampling date successfully avoided this bias, detecting up to 7 different strains per site at a given date. None of these strains were detected in our previous studies (Gaertner, *et al.*, 2011, Sha, *et al.*, 2011), however, 4 of them (S2, S10, S16, S21) were recovered from feces of deer and cattle collected in the adjacent upland during the time of the experiment (data not shown). With exception of strain S2, these were low abundance strains detected only once (S16 and S21) or twice (S10, June 21 in water and August 1 in biofilm, both from the spring arm site). While these data demonstrate a high diversity of salmonellae in a small area and the persistence of specific strains in environmental samples (e.g. viable cells of S2 in water and biofilms at different times), assumptions about the time and mode of contamination of these strains follow the same line of speculation made above for contamination of *Salmonella* in general. Future studies therefore need to address the potential role of biofilms providing protection or nutrient resources (Watnick & Kolter, 2000) which

would allow salmonellae to either persist or ultimately grow in a prevailing habitat for microorganisms including human pathogens (Watnick & Kolter, 2000, Donlan, 2002, Declerck, 2010).

Quantification of salmonellae

Quantification attempts of salmonellae by *q*PCR in samples without prior enrichment did not result in reliable detection, even though many of these samples were positive for salmonellae by end-point PCR after enrichment and thus harbor salmonellae. Both end-point PCR and *q*PCR were based on the detection of fragments of the *inv*A gene that encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by salmonellae (Suárez & Rüssmann, 1998, Khan, *et al.*, 2000), and present in all *Salmonella enterica* subspecies as well as in *S. bongori* (Malorny, *et al.*, 2003). This gene has been used as target for specific quantification of salmonellae, though with alternative amplification conditions different from ours (Fallschissel, *et al.*, 2009), different primers (Daum, *et al.*, 2002, Ahmed, *et al.*, 2009, Ahmed, *et al.*, 2012) or with different detection procedures, i.e. Taqman-based detection instead of SybrGreen-based detection (Novinscak, *et al.*, 2007, Novinscak, *et al.*, 2008). Our *q*PCR method required at least 10^3 cells per 500 ml of water or 2.56 cm^{-2} of biofilm on tiles, considering the dilution of lysates into 100 µl from which 1 µl was analyzed, and the potential loss during additional purification resulting in a mean recovery of DNA of about 10% (data not shown). Our detection limit for cells is comparable to that reported by others (Ishii, *et al.*, 2006, Ahmed, *et al.*, 2012),

although seemingly lower values have been reported when *q*PCR data were related to colony forming units (CFU), e.g. in water (Clark, *et al.*, 2011), wastewater (Shannon, *et al.*, 2007) or biosolids (Novinscak, *et al.*, 2007).

In summary, our results demonstrate that viable, highly diverse salmonellae can be detected in water and biofilms independent of runoff, with numbers, however, that did not or only occasionally surpassed 10^3 cells l^{-1} of water or 2.56 cm^{-2} of biofilm. Since estimates of infective doses for salmonellae vary significantly and study dependent reports range from 4 to 45 cells (Lehmacher, *et al.*, 1995), 10 to 100 cells (Blaser & Newman, 1982) or 10^5 cells (Blaser & Newman, 1982, Kothary & Babu, 2001), it is not likely that the long-term persistence of low numbers of salmonellae in water and biofilms documented in our study poses a direct human health concern. It is more likely that long-term persistence of certain strains will eventually result in their transfer through the food chain with potential accumulation in higher orders of the food web such as crayfish or fish (Gaertner, *et al.*, 2008, Gaertner, *et al.*, 2011). These speculations, however, will require controlled studies that enable us to quantify salmonellae as they are transferred from biofilms up through the food chain. The evidence increasingly supports a paradigm wherein salmonellae within freshwater ecosystems persist at high diversity within natural biofilms.

Acknowledgements

The authors are grateful for financial support from Texas State University-San Marcos, the Department of Biology, the Alexander-Stone endowment, and the State of Texas through the American Recovery and Reinvestment Act (ARRA).

References

[1] Ahmed W, Hodgers L, Sidhu JP & Toze S (2012) Fecal indicators and zoonotic pathogens in household drinking water taps fed from rainwater tanks in Southeast Queensland, Australia. *Applied and Environmental Microbiology* **78**: 219-226.

[2] Ahmed W, Sawant S, Huygens F, Goonetilleke A & Gardner T (2009) Prevalence and occurrence of zoonotic bacterial pathogens in surface waters determined by quantitative PCR. *Water Resources* **43**: 4918-4928.

[3] Anonymous (1995) Reptile-associated salmonellosis - selected states, 1994-1995. CDC-*Morb Mortal Wkly Rep* **44**: 347-350.

[4] Anonymous (1999) Reptile-associated salmonellosis - selected states, 1996-1998. *CDC-Morbidity and Mortality Weekly Report* **48**: 1009-1013.

[5] Arnone RD & Perdek Walling J (2007) Waterborne pathogens in urban watersheds. *Journal of Water and Health* **5**: 149-162.

[6] Blaser MJ & Newman LS (1982) A review of human salmonellosis. 1. Infective dose. *Reviews of Infectious Diseases* **4**: 1096-1106.

[7] Clark ST, Gilbride KA, Mehrvar M, Laursen AE, Bostan V, Pushchak R & McCarthy LH (2011) Evaluation of low-copy genetic targets for waterborne bacterial pathogen detection via *q*PCR. *Water Resources* **45**: 3378-3388.

[8] Cote C & Quessy S (2005) Persistence of *Escherichia coli* and *Salmonella* in surface soil following application of liquid hog manure for production of pickling cucumbers. *Journal of Food Protection* **68**: 900-905.

[9] Dallap Schaer BL, Aceto H & Rankin SC (2010) Outbreak of salmonellosis caused by *Salmonella enterica* serovar Newport MDR-AmpC in a large animal veterinary teaching hospital. *Journal of Veterinary Internal Medicine* **24**: 1138-1146.

[10] Daum LT, Barnes WJ, McAvin JC, *et al.* (2002) Real-time PCR setection of *Salmonella* in suspect foods from a gastroenteritis outbreak in Kerr county, Texas. *Journal of Clinical Microbiology* **40**: 3050-3052.

[11] Declerck P (2010) Biofilms: The environmental playground of *Legionella pneumophila*. *Environmental Microbiology* **12**: 557-566.

[12] Donlan RM (2002) Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases* **8**: 881-890.

[13] Doyle MP & Erickson MC (2006) Reducing the carriage of foodborne pathogens in livestock and poultry. *Poultry Sciences* **85**: 960-973.

[14] Fallschissel K, Kampfer P & Jackel U (2009) Direct detection of *Salmonella* cells in the air of livestock stables by real-time PCR. *The Annals of Occupational Hygiene* **53**: 859-868.

[15] Franz E, van Diepeningen AD, de Vos OJ & van Bruggen AHC (2005) Effects of cattle feeding regiment and soil management type on the fate of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in manure, manure-amended soil, and lettuce. *Applied and Environmental Microbiology* **71**: 6165-6174.

[16] Gaertner J, Hahn D, Jackson J, Forstner MRJ & Rose FL (2008a) Detection of salmonellae in captive and free-ranging turtles using enrichment culture and polymerase chain reaction. *Journal of Herpetology* **42**: 223-231.

[17] Gaertner J, Wheeler PE, Obafemi S, Valdez J, Forstner MRJ, Bonner TH & Hahn D (2008b) Detection of salmonellae in fish in a natural river system. *Journal of Aquatic Animal Health* **20**: 150-157.

[18] Gaertner JP, Forstner MRJ, Rose FL & Hahn D (2008c) Detection of salmonellae in different turtle species within a headwater spring ecosystem. *Journal of Wildlife Diseases* **44**: 519-526.

[19] Gaertner JP, Mendoza JA, Forstner MRJ & Hahn D (2011) Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. *Journal of Water and Health* **9**: 458-466.

[20] Gaertner JP, Garres T, Becker JC, Jimenez ML, Forstner MRJ & Hahn D (2009) Temporal analyses of salmonellae in a headwater spring ecosystem reveals the effect of precipitation and runoff events. *Journal of Water and Health* **7**: 115-121.

[21] Hahn D, Gaertner J, Forstner MRJ & Rose FL (2007) High resolution analysis of salmonellae from turtles within a headwater spring ecosystem. *FEMS Microbiology Ecology* **60**: 148-155.

[22] Hahn D, Amann RI, Ludwig W, Akkermans AD & Schleifer KH (1992) Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. *Journal of General Microbiology* **138**: 879-887.

[23] Haley BJ, Cole DJ & Lipp EK (2009) Distribution, diversity, and seasonality of waterborne salmonellae in a rural watershed. *Applied and Environmental Microbiology* **75**: 1248-1255.

[24] Himathongkham S, Bahari S, Riemann H & Cliver D (1999) Survivasl of *Escherichia coli* O157:H7 and *Salmonella typhimurium* in cow manure and cow manure slurry. *FEMS Microbiology Letters* **178**: 251-257.

[25] Holley R, Walkty J, Blank G, Tenuta M, Ominski K, Krause D & Ng LK (2008) Examination of *Salmonella* and *Escherichia coli* translocation from hog manure to forage, soil, and cattle grazed on the hog manure-treated pasture. *Journal of Environmental Quality* **37**: 2083-2092.

[26] International Standard Organization (1993) Detection of salmonellae (reference method). *International Standard ISO 6579.*

[27] Ishii S, Yan T, Shively DA, Byappanahalli MN, Whitman RL & Sadowsky MJ (2006) *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. *Applied and Environmental Microbiology* **72**: 4545-4553.

[28] Islam M, Morgan J, Doyle MP, Phatak SC, Millner P & Jiang XP (2004) Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Applied and Environmental Microbiology* **70**: 2497-2502.

[29] Islam M, Morgan J, Doyle MP, Phatak SC, Millner PD & Jiang X (2004) Persistence of *Salmonella enterica* serovar Typhimurium on lettuce and parsley and in soils on which they were grown in fileds treated with contaminated manure composts or irrigation water. *Foodborne Pathogens and Disease* **1**: 27-35.

[30] Johnson-Delaney CA (1996) Reptile zoonoses and threats to public health. *Reptile Medicine and Surgery*, (Mader D, ed.), pp. 20-33. WB Saunders, Philadelphia, PA.

[31] Kearny TE, Larkin MJ & Levett PN (1993) The effects of slurry storage and anaerobic digestion on survival of pathenogenic bacteria. *Journal of Applied Bacteriology* **74**: 86-93.

[32] Khan AA, Navaz MS, Khan SA & Cerniglia CE (2000) Detection of multidruge-resistant *Salmonella typhimurium* DT104 by multiplex polymerase chain reaction. *FEMS Microbiology Letters* **182**: 355-360.

[33] Kinzelman J, McLellan SL, Daniels AD, Cashin S, Singh A, Gradus S & Bagley R (2004) Non-point source pollution: Determination of replication *versus* persistence of *Escherichia coli* in surface water and sediments with correlation of levels to readily measureable environmental parameters. *Journal of Water and Health* **2**: 103-114.

[34] Kothary MH & Babu US (2001) Infective dose of foodborne pathogens in volunteers: A review. *Journal of Food Safety* **21**: 49-73.

[35] Krtinic G, Duric P & Ilic S (2010) Salmonellae in food stuffs of plant origin and their implications on human health. *European Journal of Clinical Microbiology & Infectious Diseases* **29**: 1321-1325.

[36] Lehmacher A, Bockemuhl J & Aleksic S (1995) Nationwide outbreak of human salmonellosis in Germany due to contaminated paprika and paprika-powdered potato chips. *Epidemiology and Infection* **115**: 501-511.

[37] Levantesi C, Bonadonna L, Briancesco R, Grohmann E, Toze S & Tandoi V (2012) *Salmonella* in surface and drinking water: Occurrence and water-mediated transmission. *Food Research International* **45**: 587-602.

[38] Malorny B, Hoorfar J, Bunge C & Helmuth R (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: Towards and international standard. *Applied and Environmental Microbiology* **69**: 290-296.

[39] Mermin J, Hutwanger L, Vugia D, *et al.* (2004) Reptiles, amphibians, and human *Salmonella* infection: A population-based, case-control study. *Clinical Infectious Diseases* **38**: 253-261.

[40] Natvig EE, Ingham SC, Ingham BH, Cooperband LR & Roper TR (2002) *Salmonella enterica* serovar Typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure. *Applied and Environmental Microbiology* **68**: 2737-2744.

[41] Novinscak A, Surette C & Filion M (2007) Quantification of *Salmonella* spp. in composted biosolids using a TaqMan *q*PCR assay. *Journal of Microbiological Methods* **70**: 119-126.

[42] Novinscak A, Surette C, Allain C & Filion M (2008) Application of molecular technologies to monitor the microbial content of biosolids and composted biosolids. *Water Science and Technology* **57**: 471-477.

[43] Rahn K, De Grandis SA, Clarke RC, *et al.* (1992) Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes* **6**: 271-279.

[44] Refsum T, Handeland K, Baggesen DL, Holstad G & Kapperud G (2002) Salmonellae in avian wildlife in Norway from 1969 to 2000. *Applied and Environmental Microbiology* **68**: 5595-5599.

[45] Samant S, Sha Q, Iyer A, Dhabekar P & Hahn D (2012) Quantification of *Frankia* in soils using SYBR Green based *q*PCR. *Systematic Applied Microbiology* **35**: 191-197.

[46] Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[47] Sha Q, Gunathilake A, Forstner MR & Hahn D (2011) Temporal analyses of the distribution and diversity of *Salmonella* in natural biofilms. *Systematic Applied Microbiology* **34**: 353-359.

[48] Sha Q, Vattem DA, Forstner MRJ & Hahn D (2013) Quantifying *Salmonella* population dynamics in water and biofilms. *Microbial Ecology* (in press).

[49] Shannon KE, Lee DY, Trevors JT & Beaudette LA (2007) Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment. *The Science of the Total Environment* **382**: 121-129.

[50] Slattery RN & Fahlquist L (1997) Water quality summary of the San Marcos springs riverine system, San Marcos, Texas, July-August 1994, FS-059-57. USGS, San Antonio, TX.

[51] Suárez M & Rüssmann H (1998) Molecular mechanisms of *Salmonella* invasion: The type III secretion system of the pathogenicity island 1. *Interntional Microbiology* **1**: 197-204.

[51] Vassiliadis P, Kalapothaki V, Trichopoulos D, Mavrommatti C & Serie C (1981) Improved isolation of salmonellae from naturally contaminated meat products by using Rappaport-Vassiliadis enrichment broth. *Applied and Environmental Microbiology* **42**: 615-618.

[52] Veling J, Wilpshaar H, Frankena K, Bartels C & Barkema HW (2002) Risk factors for *Salmonella enterica* subsp. *enterica* serovar Typhimurium infection on Dutch diary farms. *Preventive Veterinary Medicine* **54**: 157-168.

[53] Watnick P & Kolter R (2000) Biofilm, city of microbes. *Journal of Bacteriology* **182**: 2675-2679.

[54] Woodward DL, Khakhira R & Johnson WM (1997) Human salmonellosis associated with exotic pets. *Journal of Clinical Microbiology* **35**: 2786-2790.

CHAPTER 5

SALMONELLAE IN FISH FECES ANALYZED BY *IN SITU* HYBRIDIZATION AND QUANTITATIVE POLYMERASE CHAIN REACTION

Sha Q, Forstner MRJ, Bonner TH & Hahn D. Salmonellae in fish feces analyzed by *in situ* hybridization and quantitative polymerase chain reaction. *Journal of Aquatic and Animal Health*, (submitted).

Abstract

The potential of fish to transfer salmonellae from heterogeneous aquatic biofilms into feces was assessed in controlled aquarium studies with suckermouth catfish *Hypostomus plecostomus* and biofilms on tiles inoculated with salmonellae. The presence of fish had detectable effects on the abundance of the microbial community (i.e. higher numbers of DAPI-stained cells) in water, withdensities of about 10^5 cells ml^{-1} of water at all sampling times during the 1-week sampling period. Numbers in feces increased 10-fold during this period from about 10^6 to 10^7 cells mg^{-1} of feces. Salmonellae were detected by both quantitative polymerase chain reaction (*q*PCR) and *in situ* hybridization in water samples directly after setup, in numbers of about 10^4 cells ml^{-1} representing up to 20% of the cells of the microbial community. Numbers decreased by 3 orders of magnitude within the first 3 days of the study representing only 0.01% of the community and became undetectable after day 5. In feces, numbers initially increased to up to 6% of the cells of the community but then declined similar to population dynamics in water samples. These results suggest that *Salmonella* are not biomagnified during gut passage, and thus, fish only provide a means for translocation of this pathogen.

Introduction

Members of the genus *Salmonella* represent important zoonotic pathogens (Humphrey, 2000) that have been detected in a broad range of animal reservoirs including invertebrates, reptiles, birds, and mammals (Beach, *et al.*, 2002, Refsum, *et al.*, 2002, Hahn, *et al.*, 2007, Gaertner, *et al.*, 2011). The intestinal tract of vertebrates is

typically assumed to be the native habitat of salmonellae with feces released then contaminating terrestrial or aquatic environments (Woodward, *et al.*, 1997, Gopinath, *et al.*, 2012). Salmonellae persist in soil and water but also in plants and biofilms for extended periods (Murray, 1991, Baloda, *et al.*, 2001, Cote & Quessy, 2005, Ishii, *et al.*, 2006, Byappanahalli, *et al.*, 2009). In biofilms, for example, we detected salmonellae even in habitats of exceptional water quality, such as spring-fed Spring Lake and the upper reach of the San Marcos River, Texas (Hahn, *et al.*, 2007, Gaertner, *et al.*, 2008, Gaertner, *et al.*, 2011, Sha, *et al.*, 2011). Salmonellae were present in natural biofilms in Spring Lake with a significant micro-heterogeneity and with differences in diversity of viable strains (Sha, *et al.*, 2011). In the laboratory, specific isolates remained pathogenic, persistent, and viable in biofilm and the water column up to 28 d (Sha, *et al.*, 2013).

In the upper reach of the San Marcos River, salmonellae were detected in the intestine of four trophically diverse fishes, i.e., piscivorous largemouth bass *Micropterus salmoides*, omnivorous channel catfish *Ictalurus punctatus*, invertivorous and detritivorous common carp *Cyprinus carpio*, and algivorous and detritivorous suckermouth catfish, with up to 33% of the fish analyzed being positive for salmonellae, and serovars being highly variable among individuals (Gaertner, *et al.*, 2008). Salmonellae are not considered to be part of the normal intestinal flora of fish (Janssen & Meyers, 1968, Pal & Dasgupta, 1991), even though they were detectable for up to 30 days in catfish artificially exposed to salmonellae (Lewis,

1975). Thus, fish exposed to salmonellae could become asymptomatic carriers of this pathogen (Brunner, 1974, Bocek, *et al.*, 1992). Consequently, fish constitute an important factor potentially influencing the dissemination and persistence of salmonellae in aquatic environments (Lawton & Morse, 1980).

The aim of our study was to determine if fish would consume salmonellae from natural biofilms and return them to the environment through fecal matter, ultimately enhancing abundance or persistence of salmonellae in aquatic environments. In this study, we used the same design as in our previous studies on the fate of salmonellae in biofilms, which were conducted as controlled aquarium studies using biofilms on tiles inoculated with salmonellae (Sha, *et al.*, 2013). Suckermouth catfish was selected to assess the role of fish in the transfer of salmonellae from biofilms into feces, because of their consumption of algae and amorphous detritus from benthos of the San Marcos River (Pound et al. 2011). Quantification of salmonellae was achieved at selected sampling times during a week using quantitative polymerase chain reaction (*q*PCR) and *in situ* hybridization, and data related to shifts in abundance of the entire microbial communities in time.

Material and Methods

Heterogeneous aquatic biofilms were grown on ceramic tiles (2.2 x 2.2 cm, non-glazed) in a stream channel adjacent to the Freeman Aquatic Biology Building at Texas State University-San Marcos with running spring water for 12 months. Previous studies using more than 120 tiles with biofilms demonstrated the absence of

salmonellae (Sha, *et al.*, 2013), and therefore biofilms from only 10 haphazardly selected tiles were checked for salmonellae by PCR after semi-selective enrichment in Rappaport-Vassiliadis Broth (RVS) broth (Gaertner, *et al.*, 2009, Sha, *et al.*, 2011). Since these controls remained negative for salmonellae, all remaining biofilms were assumed to be free of salmonellae as well. Tiles with biofilms were then used in three treatments with three replicates each and established in 36 L-aquaria in the laboratory. Treatment 1 and 2 each contained 200 tiles with biofilms free of salmonellae that were placed on the bottom of each aquarium. For Treatment 3, tiles with biofilms were covered in aquaria containing 10 L of water. This water was inoculated with *Salmonella* strain S11 serovar Thompson with pulsed-field gel electrophoresis (PFGE) pattern XB-SLTH-096 [01] determined at the Texas Department of State Health Services; Austin). This strain was previously isolated from biofilms (Sha, *et al.*, 2011) and known to be pathogenic in feeding studies with the nematode *Caenorhabditis elegans* (Sha, *et al.*, 2013). Strain S11 was grown in LB medium for 16 h, washed with tap water twice, and inoculated to a final density of approximately 10^6 cells ml^{-1} estimated from the OD_{564} reading. Sixteen hours after inoculation, tiles were transferred to 3 *Salmonella*-free aquaria. Biofilms on these tiles harbored approx. $6.0 \pm 1.4 \times 10^6$ *Salmonella* cells as demonstrated by *q*PCR analysis for 9 haphazardly selected tiles (Sha, *et al.*, 2013). All aquaria were then filled with spring water, and aerated through air stones (3 cm^3). Aquaria with treatments 2 and 3 received one large or up to six small suckermouth catfish, taken

from Spring Lake by grappling. All treatments were kept at room temperature (i.e. 25°C) and artificial light conditions (16 h light and 8 h dark) photoperiod for seven days.

Water samples were collected directly after setup, whereas additional water samples and fish feces samples were obtained in 12-h intervals (i.e. 12, 24, 36, 48, 60 and 72 h after setup), followed by 24-h intervals (i.e. 4, 5, 6, and 7 d after setup). Water samples (500 ml) were filtered through 0.2 µm Whatman Nuclepore Track-Etched membranes, and the filter placed into 50 ml Falcon tubes containing 20 ml of PBS buffer (phosphate-buffered saline; 0.13M NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO4, pH 7.2). Cells were released from filters by sonication in a Fisher sonic cleaner (2QT; Fisher Scientific Inc., PA) for 10 min. Filters were removed afterwards, and released cells collected by centrifugation at 4,400 x g for 15 min (Sha*, et al.*, 2013). Fish feces (40 ml) were collected with a syringe from the bottom of each aquarium and concentrated by centrifugation at 4,400 x g for 15 min. Cell pellets from water and feces were resuspended in 1 ml of sterile distilled water, and each 3 subsamples of 100 µl then used for quantification by *in situ* hybridization or *q*PCR. After 3 and 7 d, additional 100-µl-samples were used for semi-selective enrichment and characterization of *Salmonella* isolates by rep-PCR (Hahn*, et al.*, 2007).

At the end of the study after 7 d, fish were pithed, intestines removed, and intestinal lining and contents were exposed by a longitudinal incision. Intestines from fish of the same treatment were pooled and transferred to 1 mL of distilled water in an

Eppendorf tube which was shaken by hand for 20 seconds to release and disperse the content of the intestines. After removal of intestines, the remaining liquid was filled up to 1 ml with distilled water. Each three 100-µl subsamples were then used for quantification of salmonellae by *in situ* hybridization and *q*PCR, and for semi-selective enrichment and subsequent analysis by end-point PCR.

For quantification of salmonellae by *in situ* hybridization, the subsamples of water, feces and fish intestine content were fixed in 4% paraformaldehyde in PBS at 4°C for 16 h (Amann, *et al.*, 1990). Afterwards, samples were washed in PBS and stored in a final volume of 500 µl of 50% ethanol in PBS at -20□ until further use (Amann et al., 1990). Samples were spotted on gelatin-coated slides [0.1% gelatin, 0.01% $KCr(SO_4)_2$], dried at 42ºC for 15 min, and subsequently dehydrated in 50%, 70%, and finally 95% ethanol for 3 min each. Hybridizations were carried out with probe Sal3 (5'AAT CAC TTC ACC TAC GTG, *E. coli* position 1713–1730) (Nordentoft et al., 1997) that binds to 23S rRNA of all *S. enterica* subspecies tested so far (excepting only subspecies IIIa), but should not detect *S. bongori* (Fang, *et al.*, 2003). Reactions were performed in 9 µl of hybridization buffer [0.9M NaCl, 20mM Tris/HCl, 5mM EDTA, 0.01% sodium dodecyl sulfate (SDS), pH 7.2] containing 10% formamide, to which 1 µl of probe (25 ng ml^{-1}) that included 4'6-diamidino-2-phenylindole (DAPI) at a final concentration of 200 ng ml^{-1} was added, at 42ºC for 2 h. After hybridization, the slides were washed with hybridization buffer at room temperature for 15 min, rinsed with distilled water, and air-dried. Slides were mounted with Citifluor AF1

solution (Citifluor Ltd, London, UK) and examined with a Eclipse 80i microscope, fitted for epifluorescence microscopy with a mercury lamp (X-Cite^{TM} 120; Nikon**)** and filter cubes UV-2E/C (Nikon; EX340-380, DM400, BA4435-485, for DAPI detection) and CY3 HYQ (Nikon; EX535/50, DM565, BA610/75, for Cy3 detection), respectively. Bacteria were counted at 1000 x magnification in 25 fields, selected at random, covering an area of 0.01 mm^2. DAPI and Cy3 pictures were taken from the same image using a cooled CCD camera (CoolSNAP *ES*^{2}; Photometrics, Tucson, AZ), and Nikon's NIS Elements imaging software (Version 3).

Treatment effects in the number of DAPI-stained cells in water and feces across time intervals were tested with a one-factor ANOVA (α =0.05) with Tukey's HSD used to test differences between treatments. Analyses were conducted in the software package R, version 2.11.1 (www.R-project.org).

For the quantification of salmonellae by *q*PCR, cells in the subsamples of water, feces and fish intestine content were lysed in a final volume of 200 µl of 50 mM NaOH at 65 for 30 min. Detection and quantification of salmonellae was achieved using lysates or 10-fold dilutions as template in a SYBR Green based *q*PCR performed in triplicate in a total volume of 20 µl containing 10 µl of Quanta Mix (Quanta BioSciences, Gaithersburg, MD), 0.2 µl of each primer 139 ($^{5'}$GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 ($^{5'}$TCA TCG CAC CGT CAA AGG AAC C) (100 ng µl^{-1}) and 1 µl of DNA template in an Eppendorf Mastercycler (ep realplex2; Eppendorf, Hauppauge, NY) (Sha, *et al.*, 2013). Conditions included an initial

denaturation at 96°C for 3 min, and 35 cycles of denaturation at 96°C, annealing at 64°C, and extension at 72°C, each for 30 seconds. The amplification was followed by a melting curve analysis. Quantification was based on a standard curve generated from serial dilutions of ethanol-fixed cells of *Salmonella* Typhimurium (ATCC14028) quantified by epifluorescence microscopy (Eclipse 80i; Nikon, Lewisville, TX) after DAPI staining.

Semi-selective enrichment of salmonellae was used for their detection in intestine samples by end-point PCR, and the characterization of isolates in intestine samples, and in water and feces samples collected on days 3 and 7 by rep-PCR. For enrichment, each 100 µl subsample was transferred to a 2 ml cryotube containing 1 ml of Buffered Peptone Water (BPW) (l^{-1}: 10 g peptone, 5 g NaCl, 9 g Na_2HPO4, 1.5 g KH_2PO_4, pH 7.2) (International Standard Organization, 1993) and incubated at 37ºC. After 24 h of incubation, 100 µl of each of these samples were transferred to a 2 ml cryotube containing 1 ml of Rappaport-Vassiliadis Enrichment Broth (RVS) (l^{-1}: 4.5 g peptone (soymeal), 29 g $MgCl_2$ x 7 H_2O, 8 g NaCl, 0.4 g KH_2PO_4, 0.036 g malachite-green, pH 5.2) and incubated at 37ºC for 24 h (Vassiliadis, *et al.*, 1981). Sub-samples (100 µl) of this semi-specific enrichment for salmonellae were transferred to new tubes with RVS, and salmonellae were enriched a second time as stated above (Gaertner, *et al.*, 2008).

For end-point PCR analyses of intestine contents, 100 µl samples of this second enrichment was transferred to a sterile 1.5 ml Eppendorf tube, and cells were pelleted

by centrifugation at 14,000 x g for 2 min. The cell pellet was washed with 500 µl of sterile distilled water once, and subsequently lysed in 100 µl of 50 mM NaOH by incubation at 65ºC for 15 min with shaking. Lysed cells were kept at -20ºC until use. End-point PCR was performed in a PTC-200 thermocycler (MJ Research, Waltham, MA) in a total volume of 50 µl containing 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl *Taq* polymerase (5 U μl^{-1}), and 1 µl of each primer 139 and 141 (100 ng μl^{-1}) and 1 µl of the cell lysates (Hahn, *et al.*, 2007), with an initial denaturation at 96ºC for 2 min, followed by 35 rounds of temperature cycling with denaturation at 96ºC, primer annealing at 64ºC, elongation at 72ºC, each for 30 seconds (Malorny, *et al.*, 2003). *Salmonella* Typhimurium (ATCC14028) was used as a positive control. PCR products were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 µg ml^{-1}) (Sambrook, *et al.*, 1989).

For the characterization of salmonellae in water, feces and the intestine samples, sub-samples (100 µl) of the second enrichments were plated on RVS agar (RVS solidified with 15 g agar l^{-1}). After incubation at 37ºC for 16 h, 10 colonies were chosen haphazardly from each sample and incubated in Luria–Bertani broth (LB; l^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) at 37ºC for 7 h (Sha, *et al.*, 2011). Cells from 100-µl sub-samples as well as of a culture of the inoculated *Salmonella* strain S11 were pelleted by centrifugation, and lysed in 100 µl of 50 mM NaOH as

described above. End-point PCR as described above was used to identify isolates representing salmonellae, which were then further characterized by repetitive sequence-based PCR (rep-PCR). Rep-PCR was performed in a total volume of 25 µl with primer BoxA1R ($^{5'}$CTA CGG CAA GGC GAC GCT GAC G), and 2 µl of lysate as described in (Hahn, *et al.*, 2007). Banding profiles were screened visually by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook, *et al.*, 1989), and compared to that obtained with lysed cells of *Salmonella* strain S11.

Results and Discussion

The number of DAPI-stained cells differed among treatments in water ($F_{2,6} = 7.0$; $P = 0.02$) but not in feces ($F_{1,4} = 7.0$; $P = 0.94$). In water, the number of DAPI-stained cells was lower ($P < 0.02$) in treatment 1 that did not include fish than in treatments 2 and 3 where fish were present. Across treatments, number of DAPI-stained cells ranged between 0.4 and 3.8 x 10^5 cells ml^{-1} in water and between 1.6 and 10.6 x 10^6 mg^{-1} in feces Table 5.1). These results demonstrate that fish affected the abundance of the microbial community in water samples during the experiment, most likely a consequence of permanent mixing of water through movement. The lack of time effects on the abundance of cells suggests that upwelling of precipitated cells or feces in time did not add noticible numbers of cells to the water column.

Table 5.1 DAPI-stained cells (x 10^2) in 1 mL of water or 1 mg of feces [dry weight], respectively

Time	Hours							Days			
	0	**12**	**24**	**36**	**48**	**60**	**72**	**4**	**5**	**6**	**7**
Treatment 1 (biofilm)											
Water	1541 (1370)	1016 (1322)	2865 (2594)	913 (1057)	444 (242)	582 (242)	609 (52)	1333 (1002)	777 (58)	936 (374)	983 (641)
Treatment 2 (biofilm, fish)											
Water	2228 (770)	2400 (174)	2892 (203)	2134 (1810)	1348 (1403)	872 (260)	374 (312)	986 (577)	1603 (575)	3450 (2358)	4028 (3056)
Feces	-	23376 (25407)	31627 (22492)	21961 (2666)	46627 (28794)	34150 (33694)	31544 (24068)	45955 (9229)	46276 (14799)	41233 (31914)	153610 (51476)
Treatment 3 (biofilm, fish, salmonellae)											
Water	979 (140)	3813 (2717)	3130 (2064)	3367 (1976)	987 (278)	1293 (483)	3092 (1665)	1870* (722)	2127* (1613)	2865 (373)	1681 (968)
Feces	-	16061 (15746)	21240 (10029)	42626 (8066)	36537 (16969)	23967 (12718)	32478 (12388)	72474 (26992)	67432 (312)	82964 (17704)	106223 (41769)

X ± SE, n=3)
* Values obtained from 2 aquariums instead of 3 (the aquariums with dead fish were excluded)

The interpretation of increasing numbersin feces in time is more ambiguous since the accuracy of the results is influenced potentially by methodological issues. Since samples from both water and feces were not dispersed prior to application to slides (e.g. in 0.1% pyrophosphate buffer by sonication) (Zarda, *et al.*, 1997) to avoid dilution of low numbers of salmonellae, accumulations of large numbers of cells on particulate material were noticed (Figure 5.1). These affected within-sample variability during enumeration and thus resulted in large standard errors. This issue was more pronounced in feces samples where accurate enumeration was also affected by the small amounts of feces collected and the associated difficulties to accurately determine dry weights at different times potentially resulting in an overestimation of cell numbers towards the end of the study. We were also unable to completely remove feces at each sampling which could have resulted in growth of organisms in aging

feces and thus in the detection of higher cell numbers towards the end of the study. As a consequence, we are unable to state whether the increase in numbers in feces in time is accurate or affected by our experimental setup and analyses.

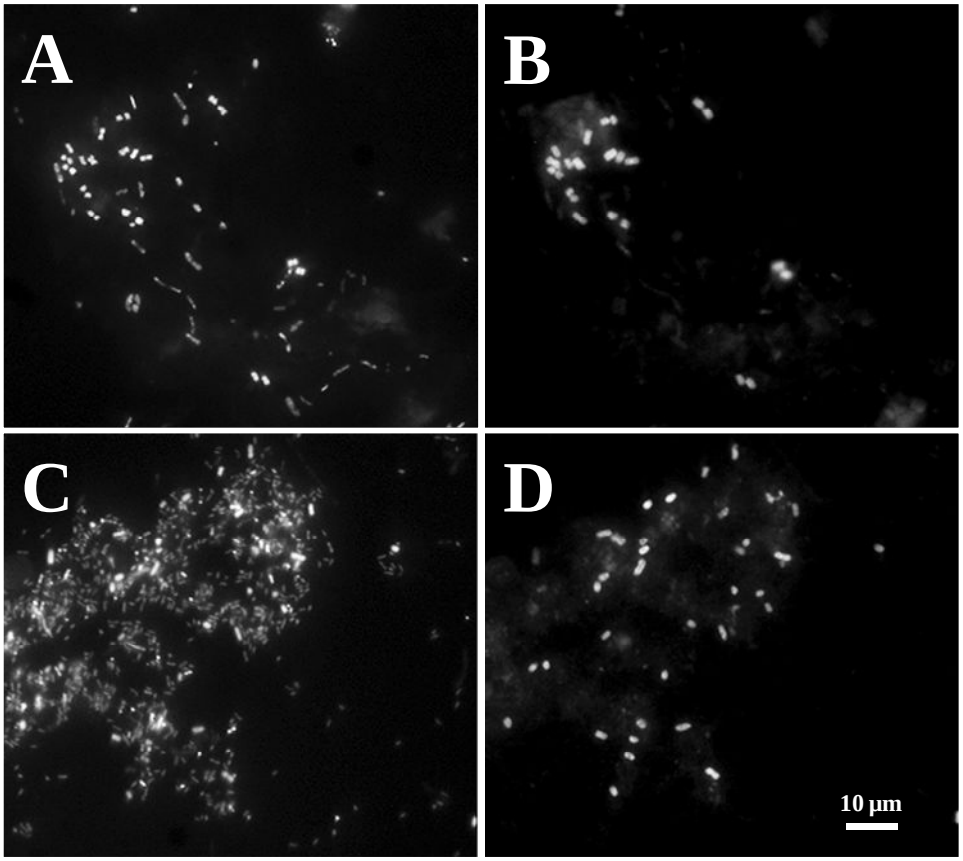


Figure 5.1 Detection of all organisms (i.e. DAPI-stained cells) (left panel) and salmonellae (right panel) in water (A and B) and feces (C and D) samples by epifluorescence microscopy.

In situ hybridization with probe Sal3 allowed us to visualize salmonellae in both water and feces samples from treatment 3 where *Salmonella* strain S11 was inoculated (Figure 5.1). Salmonellae could not be detected in intestine samples from fish harvested at the end of the study from treatment 3, and also not in any samples from treatments 1 and 2 which did not receive salmonellae (data not shown). Detection of

salmonellae in samples from treatment 3 was achieved without any pretreatments to enhance cell permeability for probes (Zarda, *et al.*, 1997), or the addition of blocking reagents to reduce potential interference of background material (Hahn, *et al.*, 1997). However, due to the small number of *Salmonella* cells present the analyses depended on our ability to concentrate cells from the original samples (i.e. cells from 500 ml of water concentrated in 1 ml of sample), and to avoid any further dilution during sample preparation for hybridization. Salmonellae were detected in water samples directly after setup, in numbers of about 10^4 cells ml^{-1}. Numbers decreased by 2 orders of magnitude within the first 72 h of the study and became undetectable after day 5 (Table 5.2). In feces samples, numbers of salmonellae increased 10-fold during the first 36 h of the experiment from 2 to 26 x 10^4 cells mg^{-1} feces and then decreased gradually to about 100-fold at day 7 (Table 5.2) and corroborated results from our previous studies (Sha, *et al.*, 2013) and studies of others (Liang, *et al.*, 1982, Klein & Alexander, 1986).

These basic *Salmonella* population dynamic profiles obtained by *in situ* hybridization were confirmed by *q*PCR analysis (Table 5.2). Regression analyses demonstrated a high correlation with R values of 0.92 and 0.89 for water and feces samples, respectively (please translate into stats language).

Table 5. 2 Number of salmonellae (x 10^2) in 1 mL of water or 1 mg of feces [dry weight], respectively

	Hours							**Days**			
	0	**12**	**24**	**36**	**48**	**60**	**72**	**4**	**5**	**6**	**7**
Detection of salmonellae by *in situ* hybridization											
Water	107 (59)	52 (30)	14 (9)	16 (7)	8 (9)	6 (0)	1 (2)	5 (6)	4 (5)	-[2]	-
Feces	nd[1]	221 (199)	543 (575)	2608 (894)	753 (379)	523 (525)	161 (151)	278 (102)	72 (124)	77 (35)	37 (63)
Detection of salmonellae by *q*PCR											
Water	216 (100)	209 (81)	65 (28)	44 (24)	-	-	-	-	-	-	-
Feces	nd	364 (120)	741 (845)	1563 (2303)	975 (1001)	33 (51)	170 (265)	16 (25)	-	27 (40)	50 (84)

(X ± SE, n=3)

[1] not sampled

[2] cell number < 100 cells

Thus, while cell numbers of the entire microbial community were either stable or slightly increased during the experiment in water and feces, respectively, numbers of *Salmonella* decreased rapidly in time. This statement is highlighted when population dynamics of salmonellae were presented as percentage of the entire community. In water samples, *Salmonella* cells were initially very prominent, representing up to 20% of the cells of the community, but then declined by one order of magnitude during each of the first three days (Figure 5.2). In feces, initial percentages were lower than in water with up to 6% of the cells of the community but also declined fast in time (Figure 5.2). These results suggest the selective removal of *Salmonella* from these samples which might be a function of predation as indicated in previous studies on salmonellae inoculated into natural or sterilized lake water (Liang, *et al.*, 1982) or

other bacteria such as *E. coli*, *Pseudomonas* sp., or *Klebsiella pneumoniae* (Scheuerman, *et al.*, 1988).

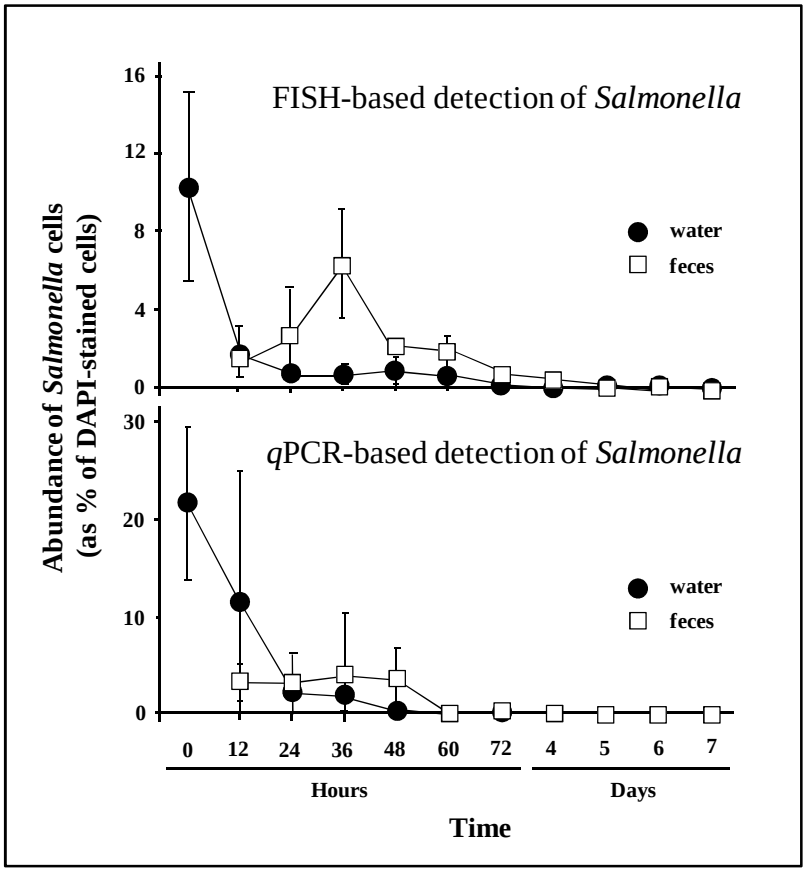


Figure 5.2 Population dynamics of salmonellae in percent of all organisms (i.e. DAPI-stained cells) in water and feces samples from treatment 3, analyzed by *in situ* hybridization (i.e. FISH-based detection) and by quantitative polymerase chain reaction (i.e. *q*PCR-based detection) in time.

Neither *in situ* hybridization nor *q*PCR analysis did detect salmonellae in intestine samples of fish harvested at the end of the study. However, end-point PCR following semi-selective enrichment of salmonellae detected them in intestine samples of all fish from treatment 3 that had received tiles with biofilms inoculated with *Salmonella* strain S11. Rep-PCR patterns of all isolates obtained from these intestines and also

from water and feces samples collected at days 3 and 7 resembled that of strain S11 indicating that this strain has been taken up and shed by the catfish. Intestines from fish of treatment 2 that had received tiles with biofilms free of salmonellae were all negative for the *inv*A gene. Salmonellae could not be isolated from these intestines, and also not from water and feces samples collected from treatment 2. These results are in agreement with those of our previous study (Gaertner, *et al.*, 2008), where we had shown that salmonellae in the intestine of fish were normally associated with particulate material, in highly variable numbers. This suggests that salmonellae are not components of the indigenous microbial community in fish intestines, but are rather taken up with particulate material, including biofilms.

Fish and other aquatic organisms have been documented as potential vectors for human pathogens for many years (Metz, 1980, Minette, 1986, Chattopadhyay, 2000, Fell, *et al.*, 2000). Infections with salmonellae are generally related to the consumption of fish (Novotny, *et al.*, 2004), but could also come from the environment contaminated by fish. Fish tank water, for example, has been reported as the source of salmonellosis in a child (Senanayake, *et al.*, 2004). Persistence and dissemination of salmonellae in fish were dependent on the number of salmonellae administered to the fish, with high numbers required for their detection in intestines or muscles of the fish 4 weeks after administration (Buras, *et al.*, 1985, Nesse, *et al.*, 2005). In our previous study (Sha, *et al.*, 2013), we have shown a fast decline of salmonellae in biofilms in time which could be basis for low percentages of

salmonellae in both water and feces samples towards the end of the study, and also explain the necessity to enrich for salmonellae cells for their detection in low numbers in the intestine. Although fish seem to be able to take up salmonellae through their food resources and shed them through their feces into the environment, numbers of salmonellae after gut passage depend on their abundance in the original food resources, and are not biomagnified during passage, and thus, fish only provide a means for translocation of this pathogen.

Acknowledgments

The authors are grateful for financial support from Texas State University-San Marcos, the Department of Biology, the Alexander-Stone endowment, and the State of Texas through the American Recovery and Reinvestment Act (ARRA). The study was performed in compliance to the rules overseen by the Texas State Institutional Animal Care and Use Committee (IACUC, permits 0721-0530-7 and 05-05C38ADFDB), and with sampling authority from the Texas Parks and Wildlife Department (TPWD, permit SPR-0601-159).

References

[1] Amann RI., Krumholz L. & Stahl DA. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *Journal of Bacteriology* **172**: 762-770.

[2] Baloda SB, Christensen L & Trajcevska S (2001) Persistence of a *Salmonella enterica* serovar typhimurium DT12 clone in a piggery and in agricultural soil amended with *Salmonella*-contaminated slurry. *Applied and Environmental Microbiology* **67**: 2859-2862.

[3] Beach JC, Murano EA & Acuff GR (2002) Prevalence of *Salmonella* and *Campylobacter* in beef cattle from transport to slaughter. *Journal of Food Protection* **65**: 1687-1693.

[4] Bocek AJ, Brady YJ & Rogers WA (1992) Exposure of Silver Carp, *Hypophthalmichthys molitrix,* to *Salmonella typhimurium*. *Aquaculture* **103**: 9-11.

[5] Brunner GH (1974) Experiments on possibilities and course of infections with *Salmonella enteritidis* and *Salmonella typhimurium* in fresh-water fish. *Zbl. Bakt. Mik. Hyg. B* **158**: 412-431.

[6] Buras N, Duek L & Niv S (1985) Reactions of fish to microorganisms in wastewater. *Applied and Environmental Microbiology* **50**: 989-995.

[7] Byappanahalli MN, Sawdey R, Ishii S, Shively DA, Ferguson JA, Whitman RL & Sadowsky MJ (2009) Seasonal stability of *Cladophora*-associated *Salmonella* in Lake Michigan watersheds. *Water Reserach* **43**: 806-808.

[8] Chattopadhyay P (2000) Fish - chatching and handling. In:Robinson R.K. (ed.):Encyclopedia of food microbiology. *Library Journal* **2**: 1547.

[9] Cote C & Quessy S (2005) Persistence of *Escherichia coli* and *Salmonella* in surface soil following application of liquid hog manure for production of pickling cucumbers. *Journal of Food Protection* **68**: 900-905.

[10] Fang Q, Brockmann S, Botzenhart K & Wiedenmann A (2003) Improved detection of *Salmonella* spp. in foods by fluorescent *in situ* hybridization with 23S rRNA probes: A comparison with conventional culture methods. *Journal of Food Protection* **66**: 723-731.

[11] Fell G, Hamouda O, Lindner R, Rehmet S, Liesegang A, Prager R, Gericke B & Petersen L(2000) An outbreak of *Salmonella blockley* infections following smoked eel consumption in Germany. *Epidemiology and Infection* **125**: 9-12.

[12] Gaertner J, Hahn D, Jackson J, Forstner MJR & Rose FL (2008) Detection of salmonellae in captive and free-ranging turtles using enrichment culture and polymerase chain reaction. *Journal of Herpetology* **42**: 223-231.

[13] Gaertner J, Wheeler PE, Obafemi S, Valdez J, Forstner MJR, Bonner TH & Hahn D (2008) Detection of salmonellae in fish in a natural river system. *Journal of Aquatic Animal Health* **20**: 150-157.

[14] Gaertner JP, Forstner MRJ, Rose FL & Hahn D (2008) Detection of salmonellae in different turtle species within a headwater spring ecosystem. *Journal of Wildlife Diseases* **44**: 519-526.

[15] Gaertner JP, Mendoza JA, Forstner MRJ & Hahn D (2011) Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. *Journal of Water Health* **9**: 458-466.

[16] Gaertner JP, Garres T, Becker JC, Jimenez ML, Forstner MRJ & Hahn D (2009) Temporal analyses of salmonellae in a headwater spring ecosystem reveals the effect of precipitation and runoff events. *Journal of Water Health* **7**: 115-121.

[17] Gopinath S, Carden S & Monack D (2012) Shedding light on *Salmonella* carriers. *Trends in Microbiology* **20**: 320-327.

[18] Hahn D, Zepp K & Zeyer J (1997) Whole cell hybridization as a tool to study *Frankia* populations in root nodules. *Physiologia Plantarum* **99**: 696-706.

[19] Hahn D, Gaertner J, Forstner MJR & Rose FL (2007) High resolution analysis of salmonellae from turtles within a headwater spring ecosystem. *FEMS Microbiology Ecology* **60**: 148-155.

[20] Humphrey T (2000) Public-health aspects of *Salmonella* infection. *Salmonella in domestic animals* (Way C & Way A, eds.), pp. 245-263. CABI Publishing, Wallingford, UK.

[21] International Standard Organization (1993) Detection of salmonellae (reference method). *International Standard ISO 6579.*

[22] Ishii S, Yan T, Shively DA, Byappanahalli MN, Whitman RL & Sadowsky MJ (2006) *Cladophora* (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. *Applied and Environmental Microbiology* **72**: 4545-4553.

[23] Janssen WA & Meyers CD (1968) Fish - serologic evidence of infection with human pathogens. *Science* **159**: 547-548.

[24] Klein TM & Alexander M (1986) Bacterial inhibitors in lake water. *Applied and Environmental Microbiology* **52**: 114-118.

[25] Lawton RL & Morse EV (1980) *Salmonella* survival in fresh-water and experimental infections in Goldfish (*Crassuis auratus*). *Journal of Environmental Science and Health A* **15**: 339-358.

[26] Lewis DH (1975) Retention of Salmonella typhimurium by certain species of fish and shrimp. *Journal of American Veterinary Medical Association* **167**: 551-552.

[27] Liang LN, Sinclair JL, Mallory LM & Alexander M (1982) Fate in model ecosystems of microbial species of potential use in genetic engineering. *Applied Environmental Microbiology* **44**: 708-714.

[28] Malorny B, Hoorfar J, Bunge C & Helmuth R (2003) Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards and international standard. *Applied and Environmental Microbiology* **69**: 290-296.

[29] Metz H (1980) Water as a vector of infection - waterborne bacteria. *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene Serie B-Umwelthygiene Krankenhaushygiene Arbeitshygiene Praventive Medizin* **172**: 255-274.

[30] Minette HP (1986) Salmonellosis in the marine-environment - a review and commentary. *International Journal of Zoonoses* **13**: 71-75.

[31] Murray CJ (1991) Salmonellae in the environment. *Revue Scientifique et Technique* **10**: 765-785.

[32] Nesse LL, Lovold T, Bergsjo B, Nordby K, Wallace C & Holstad G (2005) Persistence of orally administered *Salmonella enterica* serovars agona and Montevideo in Atlantic salmon (*Salmo salar* L.). *Journal of Food Protection* **68**: 1336-1339.

[33] Novotny L, Dvorska L, Lorencova A, Beran V & Pavlik I (2004) Fish: a potential source of bacterial pathogens for human beings. *Veterinarni Medicina* **49**: 343-358.

[34] Pal D & Dasgupta CK (1991) Interaction of some city sewage bacteria with an Indian Major Carp, *Cirrhinus mrigala*. *Journal of Aquatic Animal Health* **3**: 124-129.

[35] Pound KL, Nowlin WH, Huffman DG & Bonner TH (2011) Trophic ecology of a nonnative population of suckermouth catfishes (*Hypostomus plecostomus*) in a central Texas spring-fed stream. *Environmental Biology of Fishes* **90**:277-285.

[36] Refsum T, Handeland K, Baggesen DL, Holstad G & Kapperud G (2002) Salmonellae in avian wildlife in Norway from 1969 to 2000. *Applied and Environmental Microbiology* **68**: 5595-5599.

[37] Sambrook J, Fritsch EF & Maniatis T (1989) *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press., Cold Spring Harbor, NY.

[38] Scheuerman PR, Schmidt JP & Alexander M (1988) Factors affecting the survival and growth of bacteria introduced into lake water. *Archives of Microbiology* **150**: 320-325.

[39] Senanayake SN, Ferson MJ, Botham SJ & Belinfante RT (2004) A child with *Salmonella enterica* serotype Paratyphi B infection acquired from a fish tank. *Medical Journal of Australia* **180**: 250-250.

[40] Sha Q, Gunathilake A, Forstner MRJ & Hahn D (2011) Temporal analyses of the distribution and diversity of *Salmonella* in natural biofilms. *Systemetic and Applied Microbiology* **34**: 353-359.

[41] Sha Q, Vattem DA, Forstner MRJ & Hahn D (2013) Quantifying *Salmonella* population dynamics in water and biofilms. *Microbiology Ecology* **(in press)**.

[42] Vassiliadis P, Kalapothaki V, Trichopoulos D, Mavrommatti C & Serie C (1981) Improved isolation of salmonellae from naturally contaminated meat products by using Rappaport-Vassiliadis Enrichment Broth. *Applied and Environmental Microbiology* **42**: 615-618.

[43] Woodward DL, Khakhira R & Johnson WM (1997) Human salmonellosis associated with exotic pets. *Journal of Clinical Microbiology* **35**: 2786-2790.

[44] Zarda B, Hahn D, Chatzinotas A, Schoenhuber W, Neef A, Amann RI & Zeyer J (1997) Analysis of bacterial community structure in bulk soil by *in situ* hybridization. *Archives of Microbiology* **168**: 185-192.

CHAPTER 6

GENERAL DISCUSSION

Discussion

The above studies showed the presence of salmonellae in natural biofilms, water and animal feces in Spring Lake and San Marcos River (San Marcos, TX, USA) with high abundance, diversity and significant microheterogeneity (Chapter 2, 4). These salmonellae in natural biofilms and water could come from animal feces washed into the aquatic systems by precipitation runoffs, due to the fact that same strains have been detected from both environmental samples (i.e. natural biofilms, water) and animal feces collected from Spring Lake surrounding terrestrial area (Chapter 4). However, salmonellae contamination was not related to precipitation events as was suggested previously (Gaertner, *et al.*, 2009, Haley, *et al.*, 2009, Gaertner, *et al.*, 2011), due to the fact that same salmonellae strains had been detected constantly in a 3-month drought period in both natural biofilms and water at the same locations in the summer of 2011. This suggests that salmonellae could persist in aquatic environments by repeated colonizing biofilms from either contaminated water or biofilm detachments (Chapter 4). Thus, the paradigm of treating salmonellae as an environmental contaminant needs to be revised, and the alternative could be an ecosystem component. Several strains retrieved from environmental samples were used for the pathogenicity test and persistent experiments. The results showed that these salmonellae strains remained virulent and could persist in the aquariums for at least 4 weeks (Chapter 3). In addition to the basic aquarium experimental setup with water, biofilm and salmonellae, fish was later introduced into this system, resulting in

a detection of high salmonellae quantity in fish feces for at least 7 days. *Salmonella* were much more frequently detected from the water in the treatment with fish comparing with those without fish (Chapter 3, 5). Such results demonstrate that salmonellae could spread through food chain and persist longer in water in aquatic systems.

Both traditional enrichments and molecular techniques were utilized in these studies. Enrichments were necessary for obtaining *Salmonella* pure cultures and were reliable for detection purposes. However, it took 3 enrichments, 5 days to get a detection result and 5 enrichments, 7 days to obtain a *Salmonella* pure culture. Besides time consuming, it is also labor intensive and prone to culturing bias. In comparison, molecular techniques such as PCR, *q*PCR and *in situ* hybridization, also used in these studies, are much faster and accurate but also have their own disadvantages. PCR based techniques are usually interfered by PCR inhibitors, especially in the application of environmental samples (Tsai & Olson, 1992, Johnson, *et al.*, 1995, Marlowe, *et al.*, 1997, Sluter, *et al.*, 1997, Stults, *et al.*, 2001, Loge, *et al.*, 2002, Audemard, *et al.*, 2004, McDevitt, *et al.*, 2007, Fittipaldi, *et al.*, 2011). In order to eliminate PCR inhibitors, DNA extraction kits are commonly used before a reaction, which often results in a high cost and significant loss of DNA (Zhou, *et al.*, 1996, Miller, *et al.*, 1999, Hurt, *et al.*, 2001, Lakay, *et al.*, 2007, Lloyd, *et al.*, 2010, Samant, *et al.*, 2012, Sha, *et al.*, 2013). Because of this, the direct detection limit by *q*PCR in the environmental samples in this dissertation were about the same as those achieved

by PCR after enrichment (*q*PCR was about 100 times more sensitive than PCR in pure cultures from studies in our laboratory). In sum, regarding salmonellae quantification, enrichment method causes bias since it only counts the culturable cells (Wagner, *et al.*, 1993, Alvarez, *et al.*, 1995, Williams, *et al.*, 2012); *q*PCR counts DNA copies from both living and dead cells (Taskin, *et al.*, 2011); *in situ* hybridization is prone to artificial and sampling effects (Tyrrell, *et al.*, 2001, Wagner, *et al.*, 2003, Daims & Wagner, 2007, Donofrio, *et al.*, 2010). Therefore, one should choose different methods prudently based on specific research purposes to avoid bias and achieve the results mostly close to the truth.

Both field and laboratory control experiments were conducted in the above chapters for different purposes. Field study allows us to directly describe the natural status of the microorganisms but was also subjected to a variety of confounding environmental and artificial effects. For example, it is impossible to control the precipitation time and thus hard to study the runoff effect. Even though Spring Lake is a protected aquatic ecosystem, human activities are constantly observed in this area, such as the routine water plants cutting by heavy machines for maintaining the clarity of the water and the annual boat racing event. The heavy machine disturbance could not be neglected in ecological studies since it severely changed the stationary structure of the lake ecosystem by causing turbulence, killing plants and animals, destroying habitats for small animals and influencing microbial community structures (Sousa, 1984, Fraterrigo & Rusak, 2008, Shade, *et al.*, 2010, Shade, *et al.*, 2010, Shade, *et al.*, 2011,

Shade, *et al.*, 2012). This could possibly relate to the repeated re-colonization of salmonellae in this environment. Vessel traveling have been documented to be responsible for water pollution through leaking or cross contamination (Shikuma & Hadfield, 2010). Thus, the boat racing event occurred one day before our first sampling effort in 2011 might be an explanation for much more salmonellae being detected in 2011 than that in 2009 at Spring Lake. In order to eliminate complex environmental factors, mesocosm experiments were also conducted in this dissertation. Such experiments have the advantage in studying individual environmental factors but also often being criticized for their conclusions not applicable to natural environments (Perrin, *et al.*, 1992, Gertler, *et al.*, 2010, Gertler, *et al.*, 2012, Shade, *et al.*, 2012).

In order to quantify salmonellae cell number in natural biofilms, we used ceramic tiles with defined surface area to grow natural biofilms in an artificial stream channel. The well developed biofilms on tiles were further used both in the natural ecosystems and the aquariums. This method successfully solved the problem that accurate quantification was not possible due to uneven biofilm distribution in natural environments. The outcome was that we quantified salmonellae cell number on one square centimeter of natural biofilms for the first time.

In sum, these experiments systematically studied the ecology of salmonellae in natural biofilms and provided valuable suggestions for public health departments for pathogen control purposes.

In the future, more studies could be conducted related to the above chapters. First, the role of environmental factors were not well studied in my experiments. Several environmental factors (i.e. temperature, pH, oxidation reduction potential, dissolved oxygen level, conductivity etc.) were tested at each sampling time at each site in Chapter 4, but the results could not explain salmonellae detection differences spatially or temporally. Studies on the effects of environmental factors on the ecology of salmonellae were rarely reported except for temperature (Giaouris, *et al.*, 2005, Haley, *et al.*, 2009), thus what factors play fundamental roles in the distribution, abundance and diversity of salmonellae in natural environments remained unknown. Studies from other pathogens (i.e. *E. coli*) suggested that environmental factors such as solar radiation (Gameson & Gould, 1985, Rhodes & Kator, 1990, Daviescolley, *et al.*, 1994), predation by protozoans (Rhodes & Kator, 1990), nutrient deficiency (Na, *et al.*, 2006), effluent clarity and turbility (Curtis, *et al.*, 1992, Krogh & Robinson, 1997, Ackerman & Weisberg, 2003, Francy, *et al.*, 2006) and wave height (Francy, *et al.*, 2006) might also affect pathogenic organism's culturability in aquatic systems (Holtschlag, *et al.*, 2008). Future studies involving selecting field sites with apparent differences (the spring arm and the slough arm of Spring Lake are geographically too close in Chapter 4) in the above factors could be conducted to test these environmental factors' effects. Principal Component Analysis (PCA) could be used to determine the important environmental factors on salmonellae survival and

persistence in natural environments and followed by a risk analysis based on model prediction for pathogen control purposes.

Second, human effects should receive more attention in microbial ecology studies and could be evaluated by designed experiments. Transportation and human expansion have caused many human specific pathogens to be ubiquitous (Smith & Guegan, 2010). Water transportation has been frequently reported to be related to water pollution and contamination (Anthony & Downing, 2003, Shirodkar, *et al.*, 2010, Ho, *et al.*, 2011, Rozell & Reaven, 2012), thus a future experiment could be conducted by taking swab samples from the bottom of each boat at different sites during a boat racing event or vessel transportation process for salmonellae analysis. Accordingly, water samples need to be collected before and after boat passing. Strains of pathogenic organisms should be isolated and compared. If the same strains were retrieved from both water and boat samples after boat passing, it suggests that the boats are disseminating pathogens and thus more strict regulation rules on vessel disinfection need to be administered by public health departments.

Third, a large amount of salmonellae isolates and strains have been retrieved and characterized from the above chapters. Some of these strains have been detected much more frequently than the others (Chapter 2 and 4). But the underlying reason for the abundant strains to survive more successfully in natural biofilms remained a mystery. Studies on the strategies of surviving in harsh natural environments used by other pathogens have been illustrated before. For example, *Vibrio cholera* could alter

phenotypes (Felter, *et al.*, 1969, Dawson, *et al.*, 1981, Baker, *et al.*, 1983, Kjelleberg & Hermansson, 1984, Wai, *et al.*, 1999), attach to higher organisms such as intestinal mucosa, brush border cells, chitin (Freter, 1970, Gibbons & Vanhoute, 1971, Guentzel & Berry, 1975, Jones, *et al.*, 1976), blue crabs (*Callinectes sapidus*) (Huq, *et al.*, 1986), aquatic arthropod *Gerris spinolae* (Shukla, *et al.*, 1995) and various species of zooplankton (Huq, *et al.*, 1990, Tamplin, *et al.*, 1990, Huq, *et al.*, 1995, Islam, *et al.*, 1999) to survive in adverse natural environments; *Legionella pneumophila* on the other hand, could both associate with higher organisms (i.e. cyanobacterium *Fischerella* sp.) by using algal extracellular products as its carbon and energy sources (Tison, *et al.*, 1980) and lysed macrophages (Chandler, *et al.*, 1979). The *dot* genes of *L. pneumophila* were identified to be essential for establishing intracellular growth of *L. pneumophila* in macrophages amoebae (Gao, *et al.*, 1997, Segal & Shuman, 1999, Solomon, *et al.*, 2000, Costa, *et al.*, 2010). Future studies could also be on the investigation of *Salmonella* survival strategies in harsh natural environments, which may involve intensive gene expression assays focusing on the genes related to biofilm formation, cell structure alteration, flagella development, intracellular growth etc. by comparing the abundant and rare salmonellae strains recovered from natural environments.

References

[1] Ackerman D & Weisberg SB (2003) Relationship between rainfall and beach bacterial concentrations on Santa Monica Bay beaches. *Journal of Water and Health* **1**: 85-89.

[2] Alvarez AJ, Buttner MP & Stetzenbach LD (1995) PCR for bioaerosol monitoring: sensitivity and environmental interference. *Applied and Environmental Microbiology* **61**: 3639-3644.

[3] Anthony JL & Downing JA (2003) Physical impacts of wind and boat traffic on Clear Lake, Iowa, USA. *Lake and Reservoir Management* **19**: 1-14.

[4] Audemard C, Reece KS & Burreson EM (2004) Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. *Applied and Environmental Microbiology* **70**: 6611-6618.

[5] Baker RM, Singleton FL & Hood MA (1983) Effects of nutrient deprivation on *Vibrio cholerae*. *Applied and Environmental Microbiology* **46**: 930-940.

[6] Chandler FW, Cole RM, Hicklin MD, Blackmon JA & Callaway CS (1979) Ultrastructure of the Legionnaires; disease bacterium. A study using transmission electron-microscopy. *Annals of Internal Medicine* **90**: 642-647.

[7] Costa J, Tiago I, da Costa MS & Verissimo A (2010) Molecular evolution of *Legionella pneumophila dotA* gene, the contribution of natural environmental strains. *Environmental Microbiology* **12**: 2711-2729.

[8] Curtis TP, Mara DD & Silva SA (1992) Influence of pH, oxygen, and humic substances on ability of sunlight to damage fecal coliforms in waste stabilization pond water. *Applied and Environmental Microbiology* **58**: 1335-1343.

[9] Daviescolley RJ, Bell RG & Donnison AM (1994) Sunlight inactivation of enterococci and fecal coliforms in sewage effluent diluted in seawater. *Applied and Environmental Microbiology* **60**: 2049-2058.

[10] Dawson MP, Humphrey BA & Marshall KC (1981) Adhesion: a tactic in the survival strategy of a marine *Vibrio* during starvation. *Current Microbiology* **6**: 195-199.

[11] Felter RA, Colwell RR & Chapman GB (1969) Morphology and round body formation in *Vibrio marinus*. *Journal of Bacteriology* **99**: 326-335.

[12] Fittipaldi M, Codony F, Adrados B, Camper AK & Morato J (2011) Viable real-time PCR in environmental samples: can all data be interpreted directly? *Microbial Ecology* **61**: 7-12.

[13] Francy DS, Darner RA & Bertke EE (2006) Models for predicting recreational water quality at Lake Erie beaches: U.S. Geological Survey Scientific Investigations Report 2006 - 5192. U.S. Geological Survey.

[14] Fraterrigo JM & Rusak JA (2008) Disturbance-driven changes in the variability of ecological patterns and processes. *Ecology Letters* **11**: 756-770.

[15] Freter R (1970) Mechanism of action of intestinal antibody in experimental *Cholera* II. Antibody-mediated antibacterial reaction at the mucosal surface. *Infection and Immunity* **2**: 556-562.

[16] Gaertner JP, Mendoza JA, Forstner MRJ & Hahn D (2011) Recovery of *Salmonella* from biofilms in a headwater spring ecosystem. *Journal of Water and Health* **9**: 458-466.

[17] Gaertner JP, Garres T, Becker JC, Jimenez ML, Forstner MRJ & Hahn D (2009) Temporal analyses of salmonellae in a headwater spring ecosystem reveals the effects of precipitation and runoff events. *Journal of Water and Health* **7**: 115-121.

[18] Gameson ALH & Gould DJ (1985) Becterial motality. part 2. *in* Investigations of sewage discharges to some British coastal waters. pp. 1-72. Water research centre environment, Medmenham, United Kingdom.

[19] Gao L, Harb O & Abu Kwaik Y (1997) Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. *Infection and Immunity* **65**: 4738-4746.

[20] Gertler C, Naether DJ, Gerdts G, Malpass MC & Golyshin PN (2010) A mesocosm study of the changes in marine flagellate and ciliate communities in a crude oil bioremediation trial. *Microbial Ecology* **60**: 180-191.

[21] Gertler C, Näther DJ, Cappello S, Gerdts G, Quilliam RS, Yakimov MM & Golyshin PN (2012) Composition and dynamics of biostimulated indigenous oil-degrading microbial consortia from the Irish, North and Mediterranean Seas: a mesocosm study. *FEMS Microbiology Ecology* **81**: 520-536.

[22] Giaouris E, Chorianopoulos N & Nychas GJE (2005) Effect of temperature, pH, and water activity on biofilm formation by *Salmonella enterica* Enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *Journal of Food Protection* **68**: 2149-2154.

[23] Gibbons RJ & Vanhoute J (1971) Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. *Infection and Immunity* **3**: 567-573.

[24] Guentzel MN & Berry LJ (1975) Motility as a virulence factor for *Vibrio cholerae*. *Infection and Immunity* **11**: 890-897.

[25] Haley BJ, Cole DJ & Lipp EK (2009) Distribution, diversity, and seasonality of waterborne salmonellae in a rural watershed. *Applied and Environmental Microbiology* **75**: 1248-1255.

[26] Ho LC, Litton RM & Grant SB (2011) Anthropogenic currents and shoreline water quality in Avalon Bay, California. *Environmental Science & Technology* **45**: 2079-2085.

[27] Holtschlag DJ, Shively D, Whitman RL, Haack SK & Fogarty LR (2008) Environmental factors and flow paths related to *Escherichia coli* concentrations at two beaches on Lake St. Clair, Michigan, 2002–2005, Scientific investigations report 2008–5028. U.S. Geological Survey.

[28] Huq A, Huq SA, Grimes DJ, Obrien M, Chu KH, Capuzzo JM & Colwell RR (1986) Colonization of the gut of the blue-crab (*Callinectes sapidus*) by *Vibrio cholerae*. *Applied and Environmental Microbiology* **52**: 586-588.

[29] Huq A, Colwell RR, Rahman R, *et al.* (1990) Detection of *Vibrio cholerae* O1 in the aquatic environment by fluorescent-monoclonal antibody and culture methods. *Applied and Environmental Microbiology* **56**: 2370-2373.

[30] Huq A, Colwell RR, Chowdhury MAR, *et al.* (1995) Coexistence of *Vibriov cholerae* O1 and O139 Bengal in plankton in Bangladesh. *Lancet* **345**: 1249-1249.

[31] Hurt RA, Qiu XY, Wu LY, Roh Y, Palumbo AV, Tiedje JM & Zhou JH (2001) Simultaneous recovery of RNA and DNA from soils and sediments. *Applied and Environmental Microbiology* **67**: 4495-4503.

[32] Islam MS, Rahim Z, Alam MJ, *et al.* (1999) Association of *Vibrio cholerae* O1 with the cyanobacterium, *Anabaena sp*., elucidated by polymerase chain reaction and transmission electron microscopy. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **93**: 36-40.

[33] Johnson DW, Pieniazek NJ, Griffin DW, Misener L & Rose JB (1995) Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. *Applied and Environmental Microbiology* **61**: 3849-3855.

[34] Jones GW, Abrams GD & Freter R (1976) Adhesive properties of *Vibrio cholera*: adhesion to isolated rabbit brush-border membranes and hemagglutinating activity. *Infection and Immunity* **14**: 232-239.

[35] Kjelleberg S & Hermansson M (1984) Starvation-induced effects on bacterial surface characteristics. *Applied and Environmental Microbiology* **48**: 497-503.

[36] Krogh M & Robinson L (1997) Environmental variables and their association with faecal coliform and faecal streptococci densities at thirteen Sydney beaches. *Marine Pollution Bulletin* **33**: 239-248.

[37] Lakay FM, Botha A & Prior BA (2007) Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. *Journal of Applied Microbiology* **102**: 265-273.

[38] Lloyd KG, MacGregor BJ & Teske A (2010) Quantitative PCR methods for RNA and DNA in marine sediments: maximizing yield while overcoming inhibition. *FEMS Microbiology Ecology* **72**: 143-151.

[39] Loge FN, Thompson DE & Call DR (2002) PCR detection of specific pathogens in water: a risk-based analysis. *Environmental Science & Technology* **36**: 2754-2759.

[40] Marlowe EM, Josephson KL, Miller RM & Pepper IL (1997) A method for the detection and quantitation of PCR template in environmental samples by high performance liquid chromatography. *Journal of Microbiological Methods* **28**: 45-53.

[41] McDevitt JJ, Lees PSJ, Merz WG & Schwab KJ (2007) Inhibition of quantitative PCR analysis of fungal conidia associated with indoor air particulate matter. *Aerobiologia* **23**: 35-45.

[42] Miller DN, Bryant JE, Madsen EL & Ghiorse WC (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Applied and Environmental Microbiology* **65**: 4715-4724.

[43] Na SH, Miyanaga K, Unno H & Tanji Y (2006) The survival response of *Escherichia coli* K12 in a natural environment. *Applied Microbiology and Biotechnology* **72**: 386-392.

[44] Perrin CJ, Wilkes B & Richardson JS (1992) Stream periphyton and benthic insect responses to additions of treated and mine drainage in a continuous-flow on-site mesocosm. *Environmental Toxicology and Chemistry* **11**: 1513-1525.

[45] Rhodes MW & Kator HI (1990) Effects of sunlight and autochthonous microbiota on *Escherichia coli* survival in an estuarine environment. *Current Microbiology* **21**: 65-73.

[46] Rozell DJ & Reaven SJ (2012) Water pollution risk associated with natural gas extraction from the marcellus shale. *Risk analysis : an official publication of the Society for Risk Analysis* **32**: 1382-1393.

[47] Samant S, Sha Q, Iyer A, Dhabekar P & Hahn D (2012) Quantification of *Frankia* in soils using SYBR Green based *q*PCR. *Systematic and Applied Microbiology* **35**: 191-197.

[48] Segal G & Shuman HA (1999) *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. *Infection and Immunity* **67**: 2117-2124.

[49] Sha Q, Vattem DA, Forstner MRJ & Hahn D (2013) Quantifying *Salmonella* population dynamics in water and biofilms. *Microbial Ecology* **(in press)**.

[50] Shade A, Chiu C-Y & McMahon KD (2010) Seasonal and episodic lake mixing stimulate differential planktonic bacterial dynamics. *Microbial Ecology* **59**: 546-554.

[51] Shade A, Chiu CY & McMahon KD (2010) Differential bacterial dynamics promote emergent community robustness to lake mixing: an epilimnion to hypolimnion transplant experiment. *Environmental Microbiology* **12**: 455-466.

[52] Shade A, Read JS, Welkie DG, Kratz TK, Wu CH & McMahon KD (2011) Resistance, resilience and recovery: aquatic bacterial dynamics after water column disturbance. *Environmental Microbiology* **13**: 2752-2767.

[53] Shade A, Read JS, Youngblut ND*, et al.* (2012) Lake microbial communities are resilient after a whole-ecosystem disturbance. *ISME Journal*

[54] Shikuma NJ & Hadfield MG (2010) Marine biofilms on submerged surfaces are a reservoir for *Escherichia coli* and *Vibrio cholerae*. *Biofouling* **26**: 39-46.

[55] Shirodkar PV, Pradhan UK, Fernandes D, Haldankar SR & Rao GS (2010) Influence of anthropogenic activities on the existing environmental conditions of Kandla Creek (Gulf of Kutch). *Current Science* **98**: 815-828.

[56] Shukla BN, Singh DV & Sanyal SC (1995) Attachment of non-culturable toxigenic *Vibrio cholera* O1 and non-O1 and *Aeromonas spp*. to the aquatic arthropod *Gerris spinolae* and plants in the River Ganga, Varanasi. *FEMS Immunology and Medical Microbiology* **12**: 113-120.

[57] Sluter SD, Tzipori S & Widmer G (1997) Parameters affecting polymerase chain reaction detection of waterborne *Cryptosporidium parvum* oocysts. *Applied Microbiology and Biotechnology* **48**: 325-330.

[58] Smith KF & Guegan J-F (2010) Changing geographic distributions of human pathogens. *Annual Review of Ecology, Evolution, and Systematics, Vol 41,* (Futuyma DJ, Shafer HB & Simberloff D. eds.). pp. 231-250.

[59] Solomon JM, Rupper A, Cardelli JA & Isberg RR (2000) Intracellular growth of *Legionella pneumophila* in *Dictyostelium discoideum*, a system for genetic analysis of host-pathogen interactions. *Infection and Immunity* **68**: 2939-2947.

[60] Sousa WP (1984) The role of disturbance in natural communities. *Annual Review of Ecology and Systematics* **15**: 353-391.

[61] Stults JR, Snoeyenbos-West O, Methe B, Lovley DR & Chandler DP (2001) Application of the 5' fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. *Applied and Environmental Microbiology* **67**: 2781-2789.

[62] Tamplin ML, Gauzens AL, Huq A, Sack DA & Colwell RR (1990) Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. *Applied and Environmental Microbiology* **56**: 1977-1980.

[63] Tison DL, Pope DH, Cherry WB & Fliermans CB (1980) Growth of *Legionella pneumophila* in association with blue-green-algae (cyanobacteria). *Applied and Environmental Microbiology* **39**: 456-459.

[64] Tsai YL & Olson BH (1992) Detection of low numbers of bacterial-cells in soils and sediments by polymerase chain-reaction. *Applied and Environmental Microbiology* **58**: 754-757.

[65] Wagner M, Amann R, Lemmer H & Schleifer KH (1993) Probing activated-sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Applied and Environmental Microbiology* **59**: 1520-1525.

[66] Wai SN, Mizunoe Y & Yoshida S (1999) How *Vibrio cholerae* survive during starvation. *FEMS Microbiology Letters* **180**: 123-131.

[67] Williams LK, Sait LC, Cogan TA, Jorgensen F, Grogono-Thomas R & Humphrey TJ (2012) Enrichment culture can bias the isolation of *Campylobacter* subtypes. *Epidemiology and Infection* **140**: 1227-1235.

[68] Zhou JZ, Bruns MA & Tiedje JM (1996) DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* **62**: 316-322.

CURRICULUM VITAE

Qiong Sha (Jade) was born in 1982 in the city of Qiqihaer in northeastern China. She received a B.S. in Forestry in 2005 from the Beijing Forestry University with a minor in law. She continued to study ecology at the Botany Institute of Chinese Academy of Sciences with Dr. Xingguo Han. After completing her master's degree in 2008, she came to the United States and enrolled in the Aquatic Resources Ph.D. program under Dr. Dittmar Hahn in the Biology Department of Texas State University-San Marcos, where she studied the ecology of salmonellae in natural biofilms.

Permanent Address: No. 203 Dongsidao Road, Jianhua District

Qiqihaer City, Heilongjiang Province, Zip Code: 161000

China

This dissertation was typed by Qiong Sha.